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14. ABSTRACT
The primary goal of this project is to evaluate the efficacy of selected antibiotic regimens in an established osteomyelitis animal model of infection from MRSA, Acinetobacter baumannii, Pseudomonas aeruginosa, and Klebsiella pneumoniae. A related arm of the project at the University of Maryland-Baltimore will identify microbial gene products of the four species mentioned above with upregulated production in biofilms using two-dimensional (2D) gel electrophoresis. In addition, the PI has worked with military collaborators to publish a series of peer-reviewed manuscripts outlining guidelines for prevention and management of infections.

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Osteomyelitis; blast wounds; antibiotics; animal model

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INTRODUCTION

We evaluated the efficacy of selected antibiotic regimens in an established osteomyelitis animal model of infection from MRSA, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. We completed a related arm of the project at the University of Maryland-Baltimore to identify microbial gene products of the four species mentioned above with upregulated production in biofilms using two-dimensional (2D) gel electrophoresis. The data generated will increase our understanding of the bacterial factors involved in microbial biofilm formation and maturation and may contribute to the eventual development of a multicomponent vaccine against the four microbial pathogens.

In Year 1 of our study, we developed a mono-organism rabbit osteomyelitis model for war wounds. In Year 2, we developed a bi-, tri-, and multiple-organism complicated war wound model in the rabbit. In Year 3, we evaluated the efficacy of a number of antibiotics in this model, and reported that our results suggested that vancomycin and tigecycline alone and the combinations of tigecycline/daptomycin were not effective in the treatment of Multi-Drug Resistant Organisms (MDRO) induced war wound rabbit osteomyelitis; however, when we adjusted the treatment schedule of tigecycline in Year 4 we found protection against MDRO in an rabbit model.

Year 1. Establishing the mono-organisms rabbit osteomyelitis war wound model

KEY RESEARCH ACCOMPLISHMENTS

A. First-round of MIC/MBC testing was completed on all five antibiotic/strain combinations:

- Tigecycline with *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*.
- Levofloxacin with *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*.
- Imipenem with *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*.
- Vancomycin with *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and Acinetobacter baumannii.

  Zyvox with *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Acinetobacter baumannii*.

Tigecycline (MIC/MBC, µg/ml)	Levofloxacin (MIC/MBC, µg/ml)	Imipemen (MIC/MBC, µg/ml)	Vancomycin (MIC/MBC, µg/ml)	Zyvox (MIC/MBC, µg/ml)
0.2/0.78	3.12/6.25	25/50	50/200	200/200
0.2/0.78	3.12/6.25	25/50	50/200	200/200
0.78/6.25	12.5/25	25/50	200/200	200/200
0.2/0.78	25/50	0.39/6.25	1.56/6.25	1.56/12.5
0.2/3.12	6.25/12.5	25/50	200/200	200/200

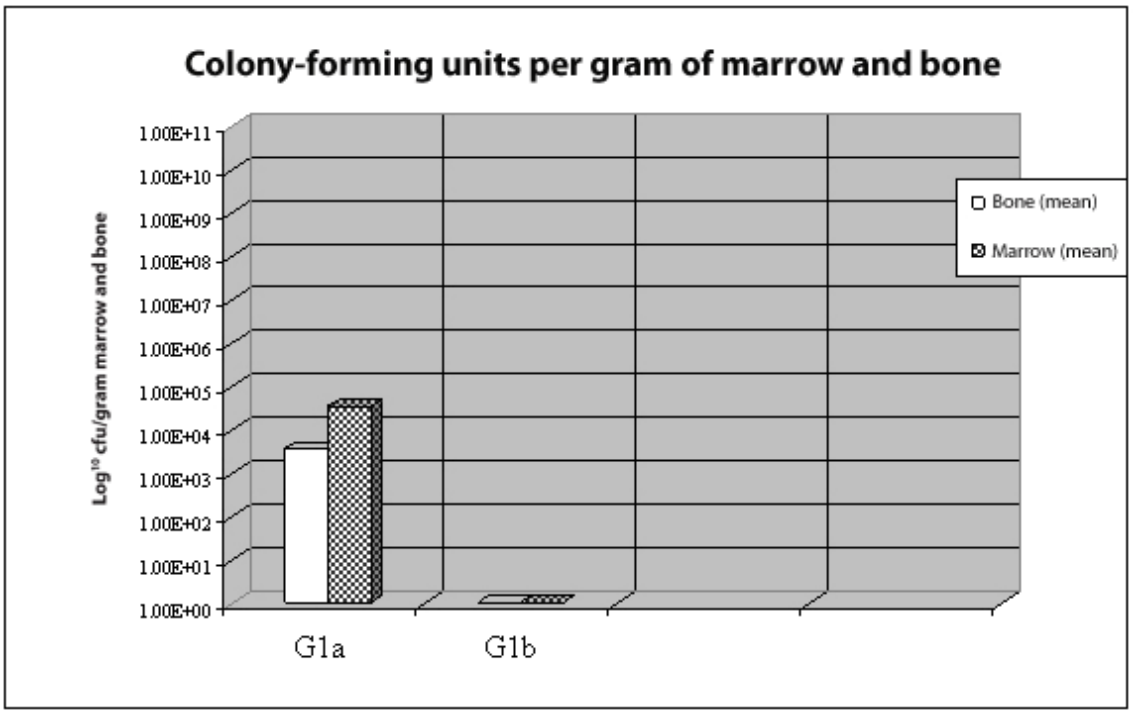
Testing was performed in August 2007 and repeated in September 2007. The chart above summarizes the results.

B. Also in the Third Quarter, we completed infections in 24 rabbits with *P. aeruginosa* (6 rabbits-1 rabbit with 105 CFU/ml, 5 rabbits with 108 CFU/ml), *Acinetobacter baumannii* (15 rabbits-5 rabbits with 108 CFU/ml, 5 rabbits with 107 CFU/ml, 5 rabbits with 105 CFU/ml) and *Klebsiella pneumonia* (3 rabbits with 105 CFU/ml) on Dec. 14, 2007. First x-rays were taken two weeks post infection (Dec. 28, 2007) and six weeks post infection (Jan. 25, 2008).

C. Initial animal model experiments (Phase I; 9 rabbits) were completed in the Fourth Quarter of grant work. Sample harvesting, bone cultures, and data analysis were performed. The results are tabulated below:

RESULTS FROM NOVEMBER 2007						
	Marrow (Mean)	**Marrow (SD)**	**Bone (Mean)**	**Bone (SD)**	***P. Aeruginosa* inoculation titer(cfu/ml)**	***P. Aeruginosa* recovered rate 8 weeks post infection**
G1a= *P. Aeruginosa*, n=5	3.42E+04	6.52E+04	3.73E+03	4.54E+03	1.00E+07	100.00%
G1b= *P. Aeruginosa*, n=4	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.00E+05	0.00%

Colony forming units were also measured at this time:



D. A review of initial data from combat-related infections was developed, working with a collaborator from the U.S. Army Institute of Surgical Research. We reported that the most frequently identified resistant strains of bacteria were *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa,* and *Acinetobacter calcoaceticus-baumannii* complex. Based on the data so far, *Acinetobacter* infections appear to be hospital-related and not from initial colonization at injury. Overuse of broad-spectrum antibiotics may be an important factor in building resistant strains. A copy of this article from *Clinical Orthopaedics and Related Research* is appended (appendix C).

E. Working with a large team of military trauma surgeons and military infectious disease specialists, the PI contributed to two articles in the *Journal of Trauma* offering guidance on war wound infections. The first, "Prevention and Management of Infections Associated With Combat-Related Extremity Injuries," made specific recommendations for pre- and post-debridement cultures, the use of antibiotics, wound irrigation, operative timing, coverage and closure of wounds, fixation of limbs, and antibiotic beads. The second, "Guidelines for the Prevention of Infection after Combat-related Injuries," reviewed care at point of injury, professional care without surgical support, care of personnel not evacuated rapidly out of the combat zone, and care with surgical support. These articles are attached in appendix C.

REPORTABLE OUTCOMES

- Establishment of baseline MIC/MBC for multiyear animal experiment
- Initial infection and examination of Phase I rabbits
- Collection of initial data (in multiyear study) of resistant infections in war wounds
- Development of guidelines for treatment of war wounds to prevent infection at multiple levels of care

CONCLUSION

The preliminary animal model experiments completed in Year One of this multiyear study provided conclusive guidance regarding the optimal use of antibiotics in wounded soldiers. Most of the work of Year One was to establish baselines in the animal model and to begin the initial infections. More definitive results began to be seen in Year Two. The combinations of pathogens and antibiotics may change in the remaining years of the experiment to reflect developments in the field as reported by our military collaborators.

The importance of guidelines for the prevention and management of military infections is perhaps best established by the large participation of military surgeons and infectious disease specialists in the effort to develop them. The medical providers of the armed forces have expressed a strong commitment to improving their response to management and prevention, particularly from resistant pathogens, as infection is associated with increases in mortality and morbidity. Improved guidance for caregivers at all levels of military medicine will help them accomplish that goal.

Year 2: Multi-organism models of wound infection; mouse biofilm project

We evaluated the efficacy of selected antibiotic regimens in an established osteomyelitis animal model of infection from MRSA, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. We began a related arm of the project at the University of Maryland-Baltimore to identify microbial gene products of the four species mentioned above with upregulated production in biofilms using two-dimensional (2D) gel electrophoresis. The data generated was expected to increase our understanding of the bacterial factors involved in microbial biofilm formation and maturation, and may contribute to the eventual development of a multi-component vaccine against the four microbial pathogens.

RESEARCH FINDINGS

Complicated osteomyelitis model (University of Missouri/The Ohio State University):

Transfer of the grant from the University of Missouri to The Ohio State University delayed initiation of work at The Ohio State University until October 2009. Work on the complicated osteomyelitis model was delayed further because of the need to obtain approval of animal use amendments requested by both The Ohio State University and ACURO. However, there were still research findings in this section of the program that need to be reported.

Mouse biofilm model (University of Maryland):

Efforts were focused on the proteomic analysis and identification of immunogenic proteins from bacterial biofilms for two additional bacterial species, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, defined in the initial proposal. Mature biofilm samples were collected from the continuous flow biofilm bioreactor system at day 14 post-inoculation for two isolates (07-001 and 07-002) of *A. baumannii* and a single isolate of *K. pneumoniae* (obtained from Jason Calhoun, The Ohio State University). Initial proteomic and immunoproteomic analyses of the *A. baumannii* isolates utilized whole cell lysate (WCL) separated by two-dimensional electrophoresis. Initial Western analysis of membrane replicates following protein transfer from the 2D gels was performed with mouse sera; an anti-biofilm humoral response to each bacterial species was generated using the mouse model of prosthetic implant infection outlined in the proposal and sera was collected at day 28 post-infection. These methods were replicated on day 14 *A. baumannii* biofilms 07-001, and resulted in few immunoreactive spots upon Western blotting. Upon alignment of the immunoreactive spots with proteins in the 2D gels, only six protein spots were found.

Due to the limited immunogenic proteins found using mouse sera, we attempted to generate a more robust anti-biofilm humoral response to *A. baumannii* and *K. pneumoniae* in a rabbit model of osteomyelitis. Briefly, the tibias of three New Zealand white (NZW) rabbits were injected with dextran beads that had been inoculated with a diluted bacterial overnight culture and incubated for two hours; this procedure was repeated for each bacterial isolate. (During this experimental study, one rabbit within the *K. pneumoniae* subset died.) Membranes were obtained from 2D gels in triplicate (*A. baumannii* 07-001 and 07-002) or duplicate (*K. pneumoniae*) using WCL from a single day 14 biofilm sample. These membranes were independently probed with rabbit serum collected 28 days post-infection. Overall, we found that these rabbits had a poor humoral response to the experimentally induced osteomyelitis infection, where a defined antibody response was displayed in 1 of 3 *A. baumannii* 07-001, 1 of 3 *A. baumannii* 07-002, and 1 of 2 *K. pneumoniae* infected animals. We concluded that the bacterial samples used in the osteomyelitis model were insufficient to establish a bacterial biofilm that was recalcitrant to clearance by the immune response. Immunogenic proteins for *A. baumannii* 07-001 and *K. pneumoniae* (18 and 24 protein spots, respectively) were defined by Western analysis using the single serum sample for each strain and sent for identification by MALDI-ToF mass spectrometry. MALDI-ToF mass spectrometry identified 12 and 15 unique putative antigenic proteins for *A. baumannii* and *K. pneumoniae*, respectively, but the vast majority of these proteins were cytoplasmic. Among these proteins, we chose the membrane-associated *A. baumannii* outer membrane protein A as well as the *K. pneumoniae* dipeptide transport protein and outer membrane protein 3a to generate recombinant proteins and confirm antigenic potential. We constructed pASK-IBA14 expression vectors encoding these genes and confirmed protein expression from all the clones. Difficulties with protein solubility hampered this portion of the project, and we addressed these issues by altering detergent types and concentrations in the lysis and purification buffers. Due to the limited serum available after the first rabbit study, we repeated the *A. baumannii* and *K. pneumoniae* infections in the rabbit model of osteomyelitis. For this study, we modified the length of co-incubation of the bacterial culture and the dextran beads from two hours to overnight. After overnight incubation, the dextran beads were spun down, resuspended in fresh culture media, and incubated for 2 hours prior to injection; we injected *A. baumannii* 07-001 and 07-002 or *K. pneumoniae* into three or four rabbits, respectively. (During this experimental study, two rabbits within the *K. pneumoniae* subset died.) We performed Western analyses using the day 35 sera from this rabbit study to probe additional biofilm WCL preparations, and confirmed all the *A. baumannii* 07-001 and 07-002 and the two remaining *K. pneumoniae* infected animals had a measurable humoral response.

KEY RESEARCH ACCOMPLISHMENTS

Overall, we found that the patterns of the immunoreactive signals for *A. baumannii* and *K. pneumoniae* aligned with the results of the initial Western analyses. We surmised that isolation and immunoproteomics analysis of the outer membranes for these Gram negative species would yield additional immunogenic, membrane-associated proteins.

REPORTABLE OUTCOMES

We identified a membrane isolation protocol that separates the inner and outer membrane fractions by sucrose gradient that has provided favorable results with a planktonic *A. baumannii* 07-001 sample. Western analysis of replicate membranes, generated by transfer of outer membrane (OM) proteins transfer from 2D gels, was completed with the four rabbit serum samples (1 sample from rabbit study 1 and 3 samples from rabbit study 2). MALDI-ToF analysis of 24 protein spots that corresponded to immunoreactive signals identified 18 planktonic *A.baumannii* proteins, including outer membrane protein A, hypothetical proteins, and proteins of known function.

Compared to the WCL protocol, we found that it was necessary to increase the biofilm sample used in this OM preparation to generate sufficient protein yield for 2D gels. We isolated the OM proteins from a day 14 *A. baumannii* 07-001 biofilm, and the immediate plans are to complete the 2D gels and Western analysis with the rabbit sera.

CONCLUSION
The resumption of Year Three rabbit studies at The Ohio State University commenced in June 2010. The pace of work was accelerated to meet the original four-year deadline specified in the original project timeline. Preliminary results were presented at a major conference.

Work under subcontract at the University of Maryland continued on schedule. We identified the antigenic membrane-associated *A. baumannii* proteins, and begin work to isolate additional recombinant proteins. If successful, we would also generate greater volumes of day 14 *A. baumannii* 07-002 and *K. pneumoniae* biofilms for isolation of OM proteins.

Year 3: Complicated osteomyelitis model (The Ohio State University):

Efficacies of Vancomycin, Tigecycline alone and the combination of Tigecycline plus Daptomycin on Multi-Drug Resistant Organism Induced War Wound Rabbit Osteomyelitis Model.

We evaluated the treatment efficacy of subcutaneous (SC) vancomycin (30 mg/kg, SC, q12h), or tigecycline (5 mg/kg, SC, q12h) alone and the combination of tigecycline (5 mg/kg, SC q12h) plus daptomycin (25 mg/kg, SC, q24h) in a rabbit model of Multi-Drug Resistant Organisms (MDRO) induced war wound osteomyelitis.

Method: Sixty NZW rabbits, 8 to 12 weeks old and weighing 2.0 to 3.5 kg, were utilized for this study. After a minimum seven-day period of acclimatization, the rabbits were placed under anesthesia and an 18-gauge needle was inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity, then sodium morrhuate (0.2 ml), infection bacteria **(**0.05 ml 10^7 *K. Pneumonia* + 0.05 ml 10^7 *A. Baumannii* + 0.05 ml 10^7 *P. Aruginosa* + 0.05ml 10^5 MRSA) and sterile saline 0.9% (0.1 ml) were injected sequentially. At two weeks post-infection, rabbits with localized proximal tibial osteomyelitis (confirmed radiographically as Grades 2-4) were separated into four Treatment Groups:

- Group A (control group, n = 15) - infected but left untreated for the duration of the study.
- Group B (vancomycin, n = 15) - rabbits were treated for four weeks with subcutaneous vancomycin, 30 mg/kg q12h. In order to ensure adequate dosing of antibiotic, peak and trough levels were obtained from six rabbits of Group B at 1 h and 12 h after administration of the first dose.
- Group C (tigecycline, n = 15) - rabbits were treated for four weeks with subcutaneous tigecycline at 5 mg/kg SC, q12h. In order to ensure adequate dosing of antibiotic, peak and trough levels were obtained from six rabbits of Group C 1 h after administration of the first dose and just before next dosage.
- Group D (tigecycline/daptomycin, n = 15) - rabbits were treated for four weeks with subcutaneous tigecycline at 5 mg/kg, q12h plus daptomycin at 25 mg/kg subcutaneously q24h. In order to ensure adequate dosing of antibiotic, peak and trough levels were obtained from six rabbits of Group D at 1 h after administration of the first dose and just before next dosage.

Quantification of bacteria in tibial matrix and marrow: Marrow and the intramedullary canal of bilateral tibiae were swabbed with sterile cotton-tip applicators; inoculated applicators were streaked onto plates of Trypticase™ Soy Agar II supplemented with 5% (v/v) defibrinated sheep blood (BBL, Becton, Dickinson & Co., Sparks, Md.), then placed into tubes containing 5 ml of Trypticase™ Soy Broth (BBL). Plates and tubes were incubated at 37°C for 24 h, and the presence or absence of growth in both media was recorded.

Marrow from each tibia from each rabbit was deposited into sterile 50 ml centrifuge tubes and weighed. Matrix from each tibia from each rabbit was cut into 0.5 cm^2 chips, placed in sterile 50 ml centrifuge tubes, and weighed. Physiological saline was added at a ratio of 3 ml of saline per g of bone matrix or marrow. Bone matrix and marrow suspensions were vortexed for 2 min and serially diluted with sterile physiological saline; 20-µl aliquots were plated onto blood agar, incubated at 37°C, and colonies counted after 24 h. The limit of detection for viable counts was 50 colony-forming units (CFUs)/ml, corresponding to 150 CFU/g of bone matrix and 200 CFU/g of marrow.

Statistical analysis of data: Means ± standard deviations were calculated using a two-tailed Student's t-test to determine whether there were significant differences in bacterial counts in matrix and marrow from left tibiae in rabbits from different groups, and to compare radiographic scores between the first and third sets of radiographs.

Mouse biofilm model (University of Maryland):
Efforts were focused on the proteomic analysis of, and identification of immunogenic proteins, from bacterial biofilms for two additional bacterial species, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, defined in the initial proposal. Mature biofilm samples were collected from the continuous flow biofilm bioreactor system at 7 hours and days 2, 7, and 14 post-inoculation for two isolates (07-001 and 07-002) of *A. baumannii* and a single isolate of *K. pneumoniae* (obtained from Jason Calhoun, The Ohio State University). Proteomic and immune-proteomic analyses of the *A. baumannii* isolates utilized whole cell lysate (WCL) separated by two-dimensional electrophoresis. Western analysis of membrane replicates following protein transfer from the 2D gels was performed sera from *A. baumannii* and *K. pneumoniae* in a rabbit model of osteomyelitis.

A localized bacteria osteomyelitis was induced percutaneously in the left lateral tibial metaphysis of all rabbits in all study groups. *Klebsiella pneumoniae* and *Acinetobacter baumannii* were grown overnight at 37°C as described in Experimental Bacteria Strain. The bacterial culture was diluted 1:100 in fresh prewarmed media (TSB) and then sterile dextran beads were added and incubated overnight. Rabbits were infected with the overnight incubated dextran beads as follows: NZW rabbits, eight to 12 weeks old and weighing 2.0 to 3.5 kg were utilized for this study. After a minimum seven-day period of acclimatization following delivery to the on-site animal resources center, rabbits were anesthetized using a subcutaneous injection of 0.3 cc of PromACE® (2%) + ketamine (45 mg/kg) and a subcutaneous injection of 0.75 cc of diazepam (5 mg/ml). Anesthetized animals were taken to a surgical suite, and the left tibia of each animal thoroughly disinfected with 70% ethanol. An 18-gauge needle was inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity. Then 5% sodium morrhuate (American Regent Laboratories, Inc., Shirley, N.Y.), infection bacteria on beads (0.1 ml), and sterile saline 0.9% were injected sequentially to each rabbit. At 0 (pre-infected control), 7, 14, 28, 42 and 56 days post-infection, and 1 ml of blood was collected from each rabbit. Rabbits were euthanized at 56 days post infection and left tibias were harvested and processed.

Gross cultures were performed for left tibias. Quantitative counts of bacteria (CFUs per gram) in left tibial bone matrix and bone marrow were determined for study groups. Left tibias from euthanized rabbits were aseptically stripped of soft tissue, placed in individual sterile 50 ml plastic centrifuge tubes, and stored at -80°C until the bones were processed.

(a) Sample Preparation: Each tibia was transferred to a sterile fume hood, the exterior of the bone was cleaned with sterile water (avoiding the needle hole in the left tibias), the proximal and distal nodules were removed and discarded, and the remaining bone broken into large fragments.

(b) Culture Preparation: The bone marrow and intramedullary canal of left tibias were swabbed with sterile cotton tip applicators for gross cultures. The inoculated applicators were streaked onto blood agar plates, then placed into 5 ml of sterile TSB. Plates and tubes were then incubated at 37°C for 24 h and growth and/or turbidity recorded.

Bone marrow recovered from left tibias were placed in sterile 50 ml plastic centrifuge tubes and weighed. Demarrowed bone matrix was broken into 0.5 cm^2 chips, placed into a sterile 50 ml centrifuge tube, and weighed. Normal sterile saline 0.9% was added in a 3:1 v/w ratio (3 ml of saline/gram of bone chips or marrow) and the suspensions were vortexed vigorously for 2 min. Six tenfold serial dilutions of each suspension were prepared with sterile normal saline 0.9%. Twenty microliter triplicate samples of the undiluted suspension as well as of each dilution were spread onto blood agar plates and incubated at 37°C for 24 h, and the bacteria concentration (CFUs per gram of bone matrix or bone marrow) calculated.

Briefly, the tibias of NZW rabbits were injected with dextran beads that had been inoculated with a diluted overnight bacterial culture and incubated overnight; this procedure was repeated for each bacterial isolate. Triplicate membranes of 2D gels, generated using WCL from multi-aged planktonic and 7 h, 2 day, and 14 day

biofilm samples, for *A. baumannii* 07-001 and 07-002 or *K. pneumoniae* were independently probed with rabbit serum collected prior to and after 7, 14, and 28 days post-infection. Overall, we found that these rabbits had an excellent humoral response to the experimentally-induced osteomyelitis infection.

Immunogenic proteins for *A. baumannii* 07-001 and *K. pneumoniae* (18 and 24 protein spots, respectively) were defined by Western analysis using the single serum sample for each strain and sent for identification by MALDI-ToF mass spectrometry. MALDI-ToF mass spectrometry identified 12 and 15 unique putative antigenic proteins for *A. baumannii* and *K. pneumoniae*, respectively, but the vast majority of these proteins were cytoplasmic. Among these proteins, we chose the membrane-associated *A. baumannii* outer membrane protein A as well as the *K. pneumoniae* dipeptide transport protein and outer membrane protein 3a to generate recombinant proteins and confirm antigenic potential. We constructed pASK-IBA14 expression vectors encoding these genes and confirmed protein expression from all the clones. Difficulties with protein solubility hampered this portion of the project, and we addressed these issues by altering detergent types and concentrations in the lysis and purification buffers.

We also developed a mouse model of prosthetic implant infection for *A. baumannii* and *K. pneumonia*. Inbred mice, C57BL/6 (6-8 weeks old), were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under micro-isolator conditions in the animal facility at the University of Maryland School of Medicine (Baltimore, MD), in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

The *A. baumannii* 07-001 and *K. pneumoniae* strains were used in these experiments. Autoclaved 0.25-mm insect pins (Fine Science Tools, Foster City, CA) were incubated for two hours in 10 ml of an overnight culture of *S. aureus* that was diluted 1:100 in sterile trypticase soy broth (TSB). Four to eight mice per experimental group received tibial implants. Mice were anesthetized via i.p. injection of 100 mg/kg ketamine (Ketaset® - Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) and 10 mg/kg xylazine (Rugby Laboratories, Inc., Rockville Center, NY). The left leg of each mouse was cleansed with povidone iodine and rinsed with 70% ethanol before surgical implantation of an *A. baumannii* 07-001 and *K. pneumoniae* -coated or uninfected control pin. All other mice did not undergo any additional treatments after surgery until sacrifice. All animal experiments were performed in accordance to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine (Baltimore, MD). Non-implanted 0.5 mm sections of pins incubated with *S. aureus* were homogenized and cultured to determine the infecting dose upon pin implantation. It was determined that approximately 2×10^5 CFU/pin (SD = 5×10^4) section were delivered to the tibia for the infection.

At 4, 7, 14, 21, 28, and 49 days post-implantation, infected and uninfected mice were euthanized, left tibiae were removed, and all soft tissue was dissected from the bone. Using sterile scissors, tibiae were cut into small pieces and placed in 300 µl of sterile 0.85% saline per 100 µg of bone. Bones were homogenized using a Polytron PT 1200 handheld homogenizer (Kinematica, Bohemia, NY) and serial 10-fold dilutions of bone homogenates were plated on sheep's blood agar plates to enumerate viable bacteria per g of bone. Additionally, 0.5 mm sections of pins representing the length inserted into the tibiae of mice were incubated with bacteria as described above and processed for culture in order to determine the infecting dose.

Using these models, the anti-biofilm humoral response to each bacterial species was generated using the mouse model of prosthetic implant infection and sera were collected at day 28 post-infection. These methods were performed on duplicate day 14 *A. baumannii* biofilms 07-001, and resulted in few immunoreactive spots upon Western blotting. Upon alignment of the immunoreactive spots with proteins in the 2D gels, only six protein spots were found. Therefore, using mouse sera for immunogenic candidates was not continued. However, this model will be used in subsequent vaccination and challenge studies

KEY RESEARCH ACCOMPLISHMENTS:

The Ohio State University:

Result: 93.33% of left tibias (14/15) from untreated control Group A showed positive cultures. The bacterial concentration in the infected tibial matrix of untreated rabbits was $7.78 \pm 4.02 \times 10^6$ CFU/g, whereas the bacterial concentration in infected tibial marrow of this group was $1.28 \pm 0.84 \times 10^6$ CFU/g. All tibias (15/15) from Group B (vancomycin treatment group) yielded positive cultures. The bacterial concentration in the infected tibial matrix was $7.21 \pm 4.84 \times 10^6$ CFU/g, whereas the bacterial concentration in infected tibial marrow of this group was $2.68 \pm 1.72 \times 10^5$ CFU/g. All tibias (13/13) from tigecycline treatment group (Group C) yielded positive cultures. The bacterial concentration in the infected tibial matrix was $8.8 \pm 3.35 \times 10^6$ CFU/g, whereas the bacterial concentration in infected tibial marrow of this group was $1.3 \pm 1.12 \times 10^7$ CFU/g. 100% tibias (13/13) in tigecycline/ daptomycin combination treatment group showed positive cultures. The bacterial concentration in the infected tibial matrix was $5.44 \pm 3.64 \times 10^7$ CFU/g, whereas the bacterial concentration in infected tibial marrow of this group was $2.94 \pm 2.54 \times 10^7$ CFU/g. Result are shown in Table 1 and Figure 1.

Radiographic results (Figure 2) reflect the rate and extent of bone reconstruction and remodeling, which in osteomyelitis always lags behind bacterial clearance. Initial radiographic scores for vancomycin treatment group (Group B) and for tigecycline treatment group (Group C) were significantly higher than for tigecycline/daptomycin group (Group D). By eight weeks after the start of treatment (two weeks after the last treatment) rabbits in the control group (Group A) showed 6.9% radiographic improvement, compared to a -16.7% mean radiographic improvement for vancomycin treatment (not significant), a -16.7% mean radiographic improvement for tigecycline treatment (not significant), and a -34.8% mean radiographic improvement for tigecycline/daptomycin treatment (significant). The mean radiographic scores of Group B and Group C were significantly different from Group A at ending point.

Nearly all rabbits treated with antibiotics exhibited symptoms of gastrointestinal distress (decreased appetite, dehydration, diarrhea, and/or weight loss). To reverse this condition, rabbits received a probiotic preparation, Probios® powder (KV Vet), 8 g po q24h, in conjunction with nutritional supplements. Weight changes of animals in Groups A-D during the eight weeks of the study are presented in Figure 3. Untreated control animals (Group A) showed the greatest mean weight gain (0.53 kg), whereas the mean weight gain by vancomycin-treated Group B (0.33 kg), Group C (-0.1 kg), and Group D (-0.2 kg). Results are shown in Figure 3.

Mouse biofilm model (University of Maryland):

Overall, we found the patterns of the immune-reactive antigens for *A. baumannii* and *K. pneumoniae*. We surmised that isolation and immune-proteomics analysis of the outer membranes for these Gram negative species may yield additional immunogenic, membrane-associated proteins. From these studies, we chose two antigens for each of *A. baumannii* and *K. pneumoniae* that were recombinantly expressed, purified, and will be used as vaccine candidates to test for protection against challenge since they match the criteria discussed below:

While the search for a single antigen that provides multimodal protection may prove successful, it seems more likely that a multicomponent vaccine will be necessary. This is the first criterion for an effective broad-range vaccine. The second is to ensure that the selected antigens are expressed in all relevant strains of the pathogen targeted by the vaccine. The genetic variation of surface-expressed proteins between strains also raises a difficulty. For this reason, it is vital to test vaccine efficacy against as large a number of strains as is realistically feasible. The third principle is to ensure that the candidate antigens are expressed in vivo throughout the infection cycle in the multiple types of infection (e.g. sepsis vs. indwelling medical device infection) for which the pathogen is the identified etiological agent. The fourth principle of antigen selection is that either (1) the selected antigen, or (2) the sum of all antigens included in a multicomponent vaccine, must be expressed through-out the infecting microbial population. Finally, the antigens selected for a biofilm vaccine must be

immunologically relevant, meaning that they must be cell-surface proteins that are visible to the humoral immune system and not obscured by the biofilm matrix.

REPORTABLE OUTCOMES:

The Ohio State University: Infection rates (Figure 1, Table 1):

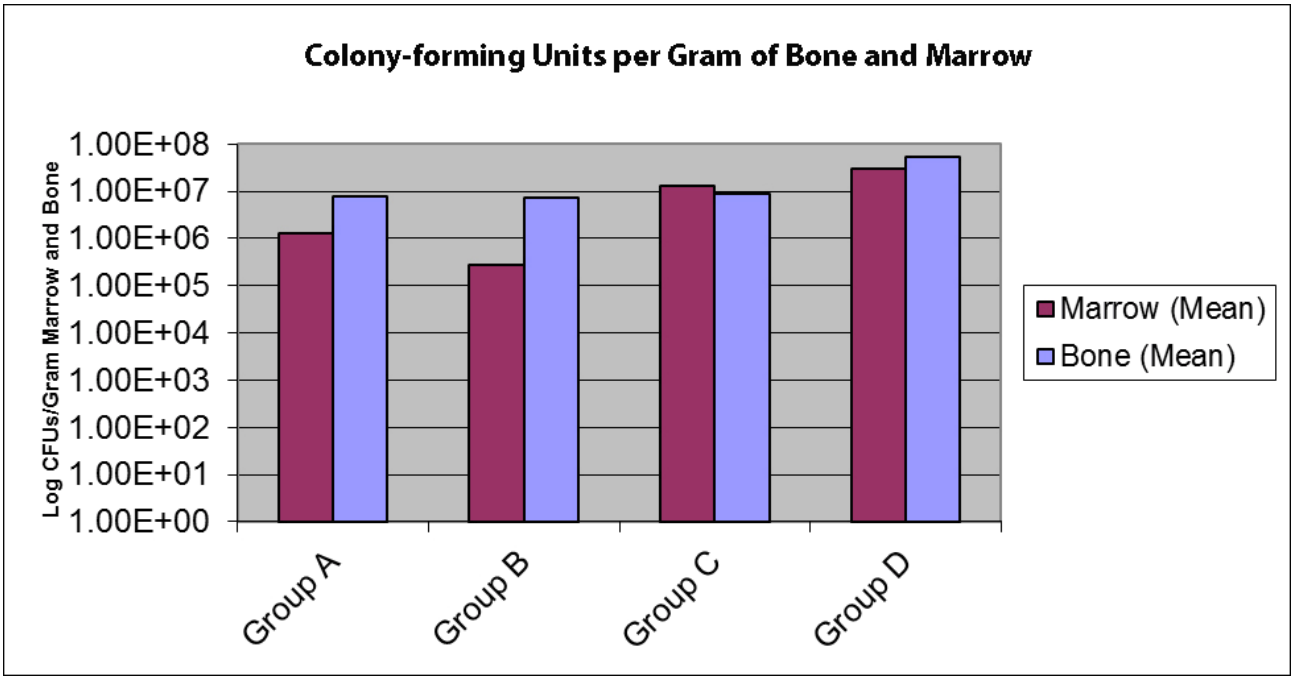


Table 1. Infection Rates

		Marrow (Mean)	Marrow (SD)	Bone (Mean)
Group A	n=15, Control	1.28E+06	8.36E+05	7.78E+06
Group B	n=15, Vancomycin (30 mg/kg, q12h)	2.68E+05	1.72E+05	7.21E+06
Group C	n=13, Tigecycline (5 mg/kg q12h)	1.30E+07	1.12E+07	8.80E+06
Group D	n=13, Tigecycline 5 mg/kg q12h/daptomycin 25 mg/kg q24h	2.94E+07	2.54E+07	5.44E+07

Figure 2: Radiographic scores

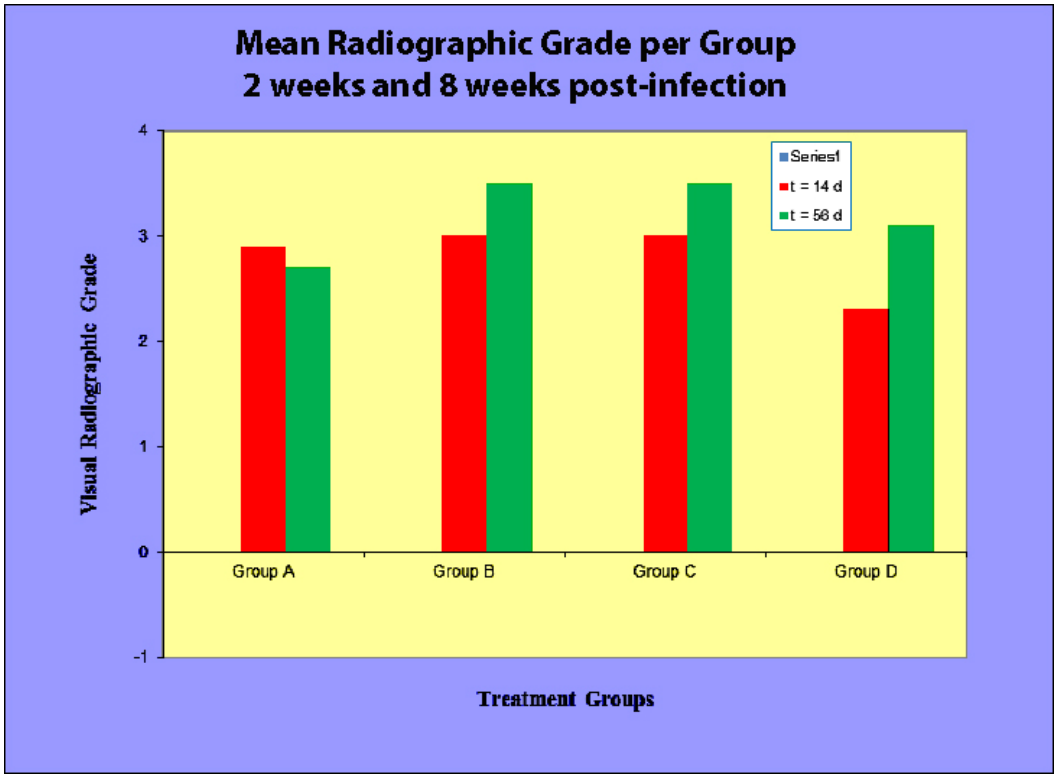
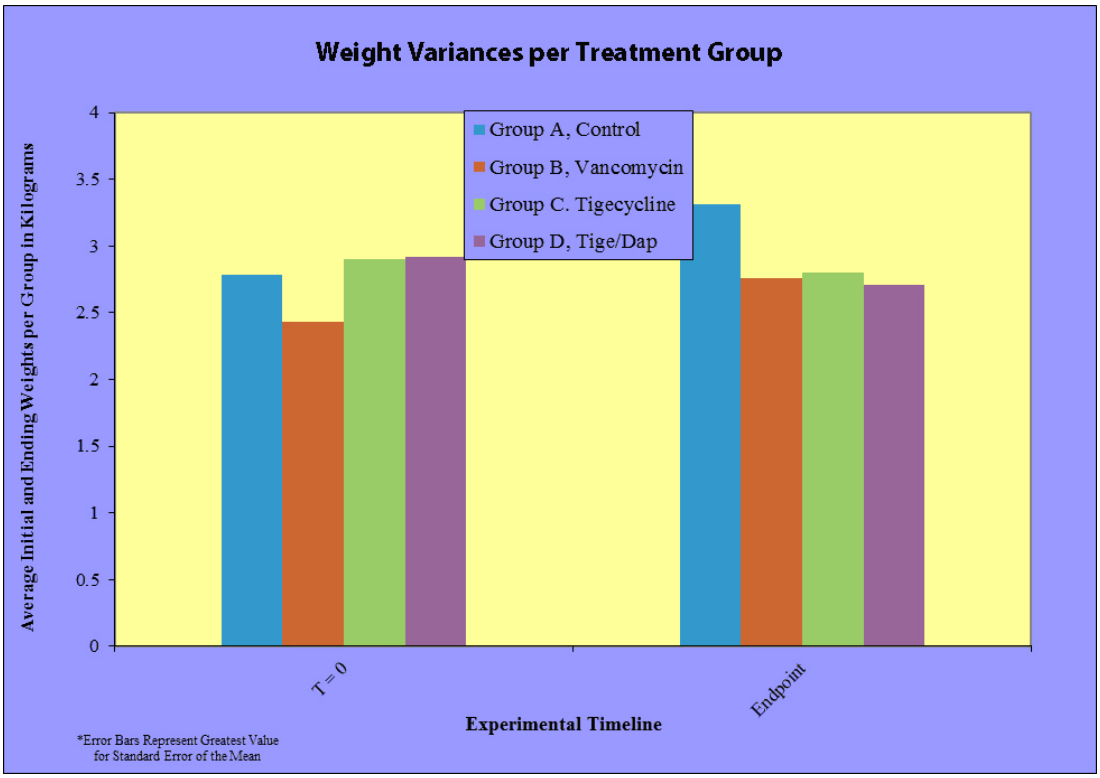


Figure 3: Weight chart



Mouse biofilm model (University of Maryland):

We utilized a membrane isolation protocol that separated the inner and outer membrane fractions by sucrose gradient that provided favorable results with a planktonic and biofilm *A. baumannii* and *K. pneumoniae*. Western analysis of replicate membranes, generated by transfer of outer membrane (OM) proteins transfer from 2D gels, was completed with the rabbit sera samples. MALDI-ToF analysis of protein spots that correspond to immunoreactive signals identified 18 *A. baumannii* proteins, including outer membrane protein A and outer membrane protein W1 and W2, as well as other hypothetical proteins, and proteins of known function. We were also able to identify a number of *K. pneumoniae* proteins, including an outer membrane protein and a dipeptide transporter. These antigens will be used in subsequent experiments to show the potential to provide protection against their respective microbial species in mouse models of prosthetic implant infection.

CONCLUSIONS

The Ohio State University: The results suggested that vancomycin and tigecycline alone and the combinations of tigecycline/daptomycin were not effective in the treatment of MDRO-induced war wound rabbit osteomyelitis.

University of Maryland: Work demonstrated potential vaccine candidates for both *A. baumannii* and *K. pneumoniae* and was tested in Year 4 for their ability to provide protection from subsequent infectious challenge in the mouse and rabbit models.

Year 4: Complicated osteomyelitis model

Prevention Efficacies of Tigecycline alone and the combination of Tigecycline Prevention plus Treatment on Multi-Drug Resistant Organism Induced War Wound Rabbit Osteomyelitis Model.

Finally, we evaluated the prevention efficacy of tigecycline (30 mg/kg, SC, once immediately post infection, 1, 3, 6, 12, 24, and 36 h post infection), tigecycline 3 h prevention plus tigecycline treatment (5 mg/kg, SC, Q12H for four weeks) on Multi-Drug Resistant Organisms (MDRO) induced war wound rabbit osteomyelitis. We reported these results below and in addition our final report on related subcontract work at the University of Maryland-Baltimore.

Method: NZW rabbits, eight to 12 weeks old and weighing 2.0 to 3.5 kg, were utilized for this study. After a minimum seven-day period of acclimatization, an 18-gauge needle was inserted percutaneously through the lateral aspect of the left tibial metaphysis into the intramedullary cavity under anesthesia, then sodium morrhuate (0.2 ml), infection bacteria **(**0.05 ml 10^7 *K. Pneumonia* + 0.05 ml 10^7 *A. Baumannii* + 0.05 ml 10^7 *P. Aruginosa* + 0.05 ml 10^5 MRSA) and sterile saline 0.9% (0.1 ml) was injected sequentially. At two weeks post-infection, rabbits with localized proximal tibial osteomyelitis (confirmed radiographically as Grades 2-4) were separated into following Treatment Groups:

- Group A (control; n = 15) - immediately post-infection, rabbits left untreated for the entire study.
- Group F (0 h prevention; n = 5) - immediately post-infection, rabbits to be treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group G (1 h prevention; n = 5) - 1 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group H (3 h prevention; n = 5) - 3 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group I (6 h prevention; n = 5) - 6 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group J (12 h prevention; n = 5) - 12 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group K (24 h prevention; n = 4) - 24 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one

subcutaneous dose.

- Group L (36 h prevention; n = 4) - 36 h post-infection, rabbits treated with tigecycline at 30 mg/kg for one subcutaneous dose.
- Group S (3 h prevention treatment; n = 11) - infected rabbits treated with one dose of tigecycline at 30 mg/kg subcutaneously (at 3 h post infection). At two weeks post-infection, rabbits were treated for four weeks with subcutaneous tigecycline at 5 mg/kg SC, q12h.

Radiographs of tibias were taken at two weeks, six weeks and eight weeks post-infection. Rabbits were anesthetized with 0.6 mg of PromACE® + ketamine (45 mg/kg) administered subcutaneously prior to radiological testing. Radiographs were scored on a scale of 0 to 4+ by three investigators and the scores averaged. Rabbits were euthanized at eight weeks post infection. Left tibias from all rabbits were harvested. Concentrations of bacteria in bone matrix and bone marrow were determined.

Quantification of bacteria in tibial matrix and marrow: Marrow and the intramedullary canal of bilateral tibiae were swabbed with sterile cotton-tip applicators; inoculated applicators were streaked onto plates of Trypticase™ Soy Agar II supplemented with 5% (v/v) defibrinated sheep blood (BBL, Becton, Dickinson & Co., Sparks, Md.), then placed into tubes containing 5 ml of Trypticase™ Soy Broth (BBL). Plates and tubes were incubated at 37°C for 24 h, and the presence or absence of growth in both media was recorded. Marrow from each rabbit tibia was deposited into sterile 50 ml centrifuge tubes and weighed. Matrix from each rabbit tibia was cut into 0.5 cm^2 chips, placed in sterile 50 ml centrifuge tubes, and weighed. Physiological saline was added at a ratio of 3 ml of saline per gram of bone matrix or marrow. Bone matrix and marrow suspensions were vortexed for 2 min and serially diluted with sterile physiological saline; 20 µl aliquots were plated onto blood agar, incubated at 37°C, and colonies counted after 24 h. The limit of detection for viable counts was 50 colony-forming units (CFUs)/ml, corresponding to 150 CFU/g of bone matrix and 200 CFU/g of marrow.

Statistical analysis of data: Means ± standard deviations were calculated using a two-tailed Student's t-test to determine whether there were significant differences in bacterial counts in matrix and marrow from left tibiae in rabbits from different groups. Fisher's exact test was used to determine the significance of bacteria clearance rate among different treatment groups. Differences between groups were deemed statistically significant if $p \leq 0.05$.

KEY RESEARCH ACCOMPLISHMENTS:

Infection Clearance Rate and recovered bacteria concentration: 6.7% of left tibias (1/15) from untreated control Group A showed infection clearance. The bacterial concentration in the infected tibial matrix of untreated rabbits was $(7.78 \pm 9.61) \times 10^6$ CFU/g. The bacterial concentration in infected tibial marrow of this group was $(1.28 \pm 3.24) \times 10^6$ CFU/g.

Five of 11 (45.4%) tibias from Group S showed infection clearance. The infection clearance between the Group A and Group S were statistically significant ($p<0.03$). The bacterial concentration of the infected tibial matrix in Group S was $(1.41 \pm 2.49) \times 10^4$ CFU/g, which was significantly lower than that of the Group A and L ($p<0.05$). The bacterial concentration in infected tibial marrow of Group S was $(0.859 \pm 1.99) \times 10^4$ CFU/g.

Two of five (40%) tibias from Group F showed infection clearance. The bacterial concentration in the infected tibial matrix was $(3.99 \pm 8.9) \times 10^7$ CFU/g, whereas the bacterial concentration in infected tibial marrow of this group was $(1.07 \pm 2.38) \times 10^5$ CFU/g. 40% tibias (2/5) in Group G showed infection clearance. The bacterial concentration in the infected tibial matrix was $(2.97 \pm 4.97) \times 10^5$ CFU/g, which was significantly lower than that of the Group A ($p<0.05$). The bacterial concentration in infected tibial marrow of Group G was $(8.05 \pm 15.4) \times 10^4$ CFU/g. 80% tibias (4/5) in Group H showed infection clearance. The infection clearance difference between the Group H and Group A and J was statistically significant ($p<0.05$). The bacterial concentration in the infected tibial matrix of Group H was $(4.64 \pm 10.4) \times 10^4$ CFU/g, which was significantly lower than that of the Groups A, I, and L ($p<0.05$). The bacterial concentration in infected tibial marrow of Group H was $(3.96 \pm 8.85) \times 10^4$ CFU/g. 60% tibias (3/5) in Group I showed infection clearance. The infection clearance difference

between the Group I and Group A was statistically significant ($p<0.03$). The bacterial concentration in the infected tibial matrix of Group I was (29.7 ± 4.96) x 10^4 CFU/g, which was significantly lower than that of the Group A, K and S ($p<0.05$). The bacterial concentration in infected tibial marrow of Group I was 4.00 ± 8.94 x 10^3 CFU/g. 0% tibias (0/5) in Group J showed infection clearance. The bacterial concentration in the infected tibial matrix of Group J was (5.80 ± 145) x 10^4 CFU/g, which was significantly lower than that of the Group A ($p<0.05$). The bacterial concentration in infected tibial marrow of Group J was (8.81 ± 19.7) x 10^4 CFU/g. 50% tibias (2/4) in Group K showed infection clearance. The bacterial concentration in the infected tibial matrix of Group K was (5.80 ± 10.8) x 10^4 CFU/g, which was significantly lower than that of the Group A and L ($p<0.05$). The bacterial concentration in infected tibial marrow of Group K was (1.52 ± 3.03) x 10^4 CFU/g. 25% tibias (1/4) in Group L showed infection clearance. The bacterial concentration in the infected tibial matrix of Group L was (29.7 ± 1.42) x 10^4 CFU/g, which was significantly lower than that of the Group A ($p<0.05$). The bacterial concentration in infected tibial marrow of Group L was (2.55 ± 3.19) x 10^4 CFU/g.

The recovered bacteria from tibia and marrow culture were identified to be *K. pneumonia* and *P. aeruginosa*. Please refer to Figures 1-2 and Table 1 for infection clearance rate and recovered bacteria concentration among all groups.

Radiographic Change: Radiographic results reflect the rate and extent of bone reconstruction and remodeling, which in osteomyelitis always lags behind bacterial clearance (Figure 3). Initial radiographic scores for tigecycline prevention groups (Groups F, G, H, I, J and K) were significantly lower than that for non treatment control group (Group A). Initial radiographic scores for tigecycline prevention groups (Groups F, G, H, I and J) were significantly lower than that for tigecycline prevention/treatment group (Group S). Initial radiographic scores for tigecycline prevention Group H (3 h post infection) were significantly lower than that for tigecycline prevention Group I (6 h post infection). By eight weeks after start of treatment (two weeks after the last treatment) rabbits in the control group (Group A) showed 6.9% radiographic improvement, compared to a -257.1% mean radiographic improvement for Group F (significant), a 8.3% mean radiographic improvement for Group G (not significant), and a -250% mean radiographic improvement for Group H (significant), a -33.3% for group I (not significant), a -47.1% for Group J (not significant), a 13.3% for group K (not significant), a 7.7% for Group L (not significant) and 51.7% mean radiographic improvement for tigecycline prevention/treatment Group S (significant). The mean radiographic scores of Groups G, H, L and S were significantly different from Group A at ending point.

Adverse Events: Of the 63 rabbits that were infected, a total of four died before completion of treatment. Two rabbits in the tigecycline prevention/treatment Group S died from a handling accident at day 5 of treatment. One rabbit in Group S was removed from study due to weight loss at day 10. Another rabbit in Group S was removed on day 42 due to anesthesia intolerance. All rabbits were monitored weekly for weight variance. The control group and all of the treatment groups showed significant weight gains for the study. Group J showed the most mean gain (0.78 ± 0.14 kg), Group I the second (0.74 ± 0.14 kg), Group L the third (0.72 ± 0.15 kg), Group K the fourth (0.67 ± 0.10 kg), Group A (control) the fifth (0.53 ± 0.17 kg), Group S the sixth (0.5 ± 0.36 kg), Group H the seventh (0.45 ± 0.15 kg), Group F the eighth (0.24 ± 0.17 kg), Group G the ninth (0.18 ± 0.23 kg). Figure 4 shows the weight changes among all groups.

REPORTABLE OUTCOMES:

Infection rates are summarized in Figure 1 and Table 1:

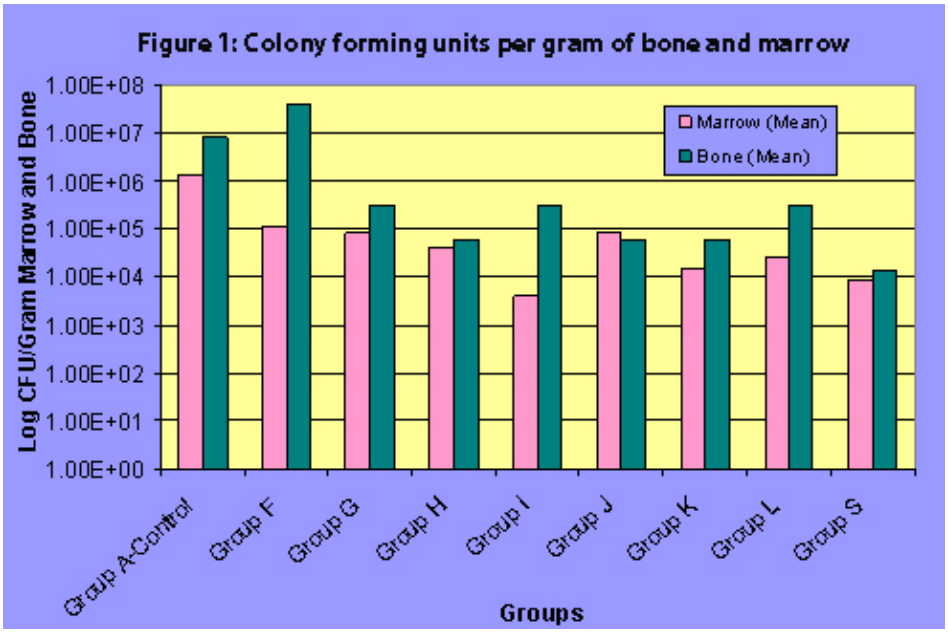


Table 1. Bacteria Load in Bone Matrix and Bone Marrow					
Groups	**Marrow CFU/g**	**Marrow SD**	**Bone CFU/g**	**Bone SD**	**infection clearance rate**
Group A, Control, n=15	**1.28E+06**	**3.24E+06**	**7.78E+06**	**9.61E+07**	**6.7% or 1/15**
Group F, T= 0 h, n=5	**1.07E+05**	**2.38E+05**	**3.99E+07**	**8.90E+07**	**40% or 2/5**
Group G, T= 1 h, n=5	**8.05E+04**	**1.54E+05**	**2.97E+05**	**4.79E+05**	**40% or 2/5**
Group H, T= 3 h, n=5	**3.96E+04**	**8.85E+04**	**4.64E+04**	**1.04E+05**	**80% or 4/5**
Group I, T= 6 h, n=5	**4.00E+03**	**8.94E+03**	**2.97E+05**	**4.96E+04**	**60% or 3/5**
Group J, T= 12 h, n=5	**8.81E+04**	**1.97E+05**	**5.80E+04**	**1.45E+06**	**0% or 0/5**
Group K, T= 24 h, n=4	**1.52E+04**	**3.03E+04**	**5.80E+04**	**1.08E+05**	**50% or 2/4**
Group L, T= 36 h, n=4	**2.55E+04**	**3.19E+04**	**2.97E+05**	**1.42E+04**	**25% or 1/4**
Group S, (3 h pre/Treat), n=11	**8.59E+03**	**1.99E+04**	**1.41E+04**	**2.49E+04**	**45.4% or 5/11**

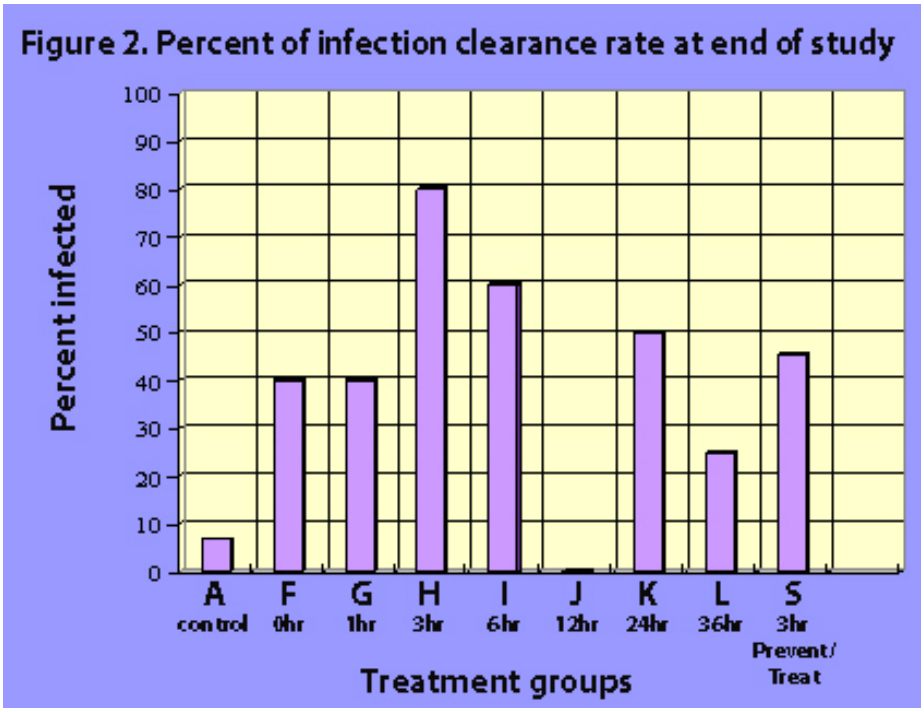


Figure 2. Percent of infection clearance rate at end of study
Percent infected
100
90
80
70
60
50
40
30
20
10
0
A control
F 0hr
G 1hr
H 3hr
I 6hr
J 12hr
K 24hr
L 36hr
S 3hr Prevent/ Treat
Treatment groups

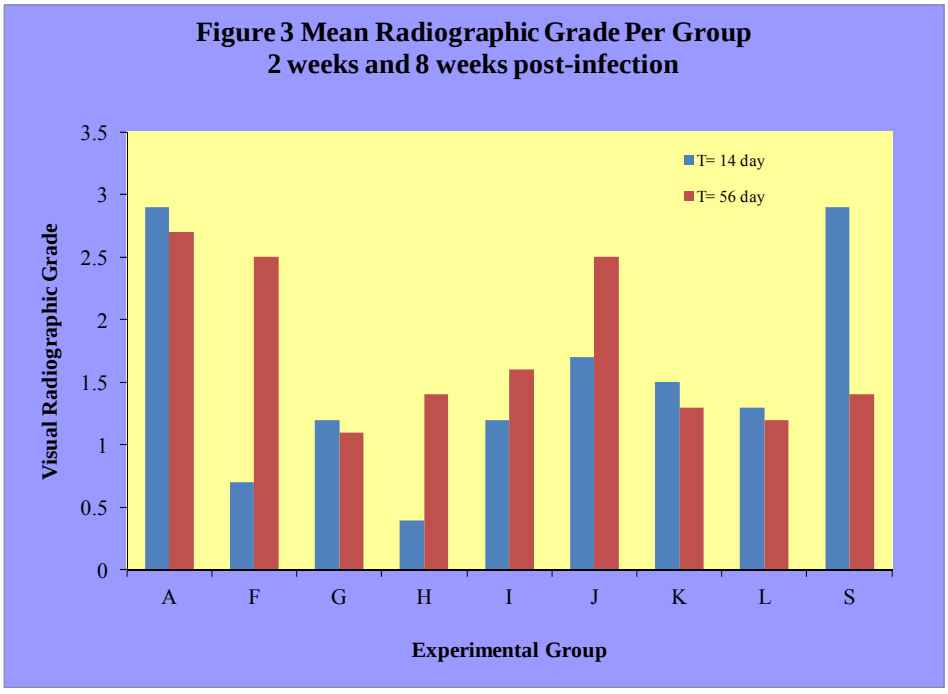
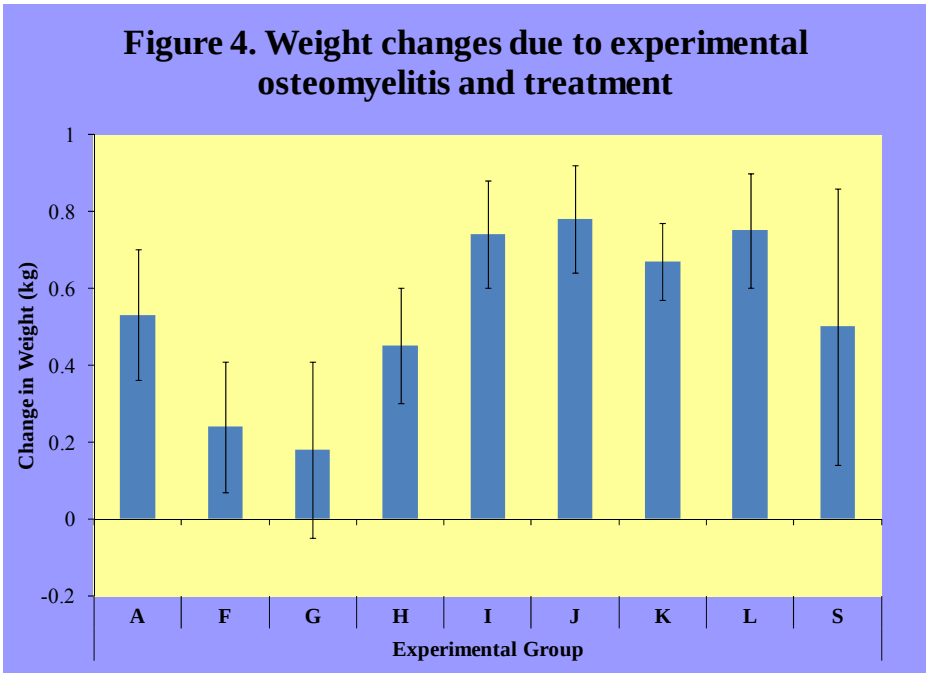


Figure 3 Mean Radiographic Grade Per Group
2 weeks and 8 weeks post-infection
Visual Radiographic Grade
3.5
3
2.5
2
1.5
1
0.5
0
T= 14 day
T= 56 day
A
F
G
H
I
J
K
L
S
Experimental Group



CONCLUSIONS (Years 1-4)

The Ohio State University: Our previous reported results suggested that vancomycin and tigecycline alone, and the combinations of tigecycline/daptomycin were not effective in the treatment of MDRO-induced war wound rabbit osteomyelitis. Our final work suggests that tigecycline alone was effective in our rabbit model of MDRO-induced war wound rabbit osteomyelitis if used in larger doses and given at different times than in our previous work (Year 3). The combination of tigecycline prevention plus treatment was effective in the treatment of MDRO-induced war wound rabbit osteomyelitis.

University of Maryland: Work to test potential vaccine candidates for both *A. baumannii* and *K. pneumoniae* and will be conducted in the coming months for their ability to provide protection from subsequent infectious challenge in the mouse and rabbit models.

REFERENCES

None.

APPENDICES

A. List of individuals receiving pay from this project.

B. List of publications related to this project during the reporting period.

C. Copies of publications listed in Appendix B.

A. List of Individuals Receiving Payment for this Project (entire reporting period)

1. Jason H. Calhoun, M.D., F.A.C.S., Chairman, Department of Orthopaedics, The Ohio State University, 725 Prior Hall, 376 W. 10th Ave., Columbus, OH 43210
2. Li-Yan Yin, M.D., Clinical Research Manager, Department of Orthopaedics, The Ohio State University, 725 Prior Hall, 376 W. 10th Ave., Columbus, OH 43210
3. Hongpeng Liu, Research Assistant, Department of Orthopaedics, The Ohio State University, 725 Prior Hall, 376 W. 10th Ave., Columbus, OH 43210
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5. Randy Roberts, Research Assistant, Department of Orthopaedics, The Ohio State University, 725 Prior Hall, 376 W. 10th Ave., Columbus, OH 43210
6. Mark E. Shirtliff, Ph.D., Assistant Professor, Department of Microbial Pathogenesis, Dental School, University of Maryland-Baltimore, 650 W. Baltimore Street, Baltimore, Maryland 21201
7. Janette Harro, Ph.D., postdoctoral assistant, Department of Microbial Pathogenesis, Dental School, University of Maryland-Baltimore, 650 W. Baltimore Street, Baltimore, Maryland 21201
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9. Brett D. Crist, M.D., Assistant Professor, Department of Orthopaedic Surgery, University of Missouri-Columbia, 213 McHaney Hall, Columbia, MO 65212
10. Gregory J. Della Rocca, M.D., Ph.D., Assistant Professor, Department of Orthopaedic Surgery, University of Missouri-Columbia, 213 McHaney Hall, Columbia, MO 65212
11. Xiuli Fan, Senior Research Lab Technician, Department of Orthopaedic Surgery, University of Missouri-Columbia, 213 McHaney Hall, Columbia, MO 65212
12. Yuening Huang, Senior Research Lab Technician, Department of Orthopaedic Surgery, University of Missouri-Columbia, 213 McHaney Hall, Columbia, MO 65212
13. Leah Briggs, Grants/Contracts Specialist, Department of Orthopaedic Surgery, University of Missouri-Columbia, 213 McHaney Hall, Columbia, MO 65212

B. List of Publications and Presentations Related to this Project

Publications (in print or preparing for publication; attachments in Appendix C):

1. Brady RA, O'May GA, Leid JG, Prior ML, Costerton JW, **Shirtliff ME**. Resolution of Staphylococcus aureus biofilm infection using vaccination and antibiotic treatment. *Infect Immun*. 2011 Apr;79(4):1797-803.

2. D'Avignon LC, Chung KK, Saffle JR, Renz EM, Cancio LC, **Prevention of Combat-Related Infections Guideline Panel**. Prevention of infections associated with combat-related burn injuries. *J Trauma*. In press.

3. Forgione MA, Moores LE, Wortmann GW, **Prevention of Combat-Related Infections Guideline Panel**. Prevention of infections associated with combat-related central nervous system injuries. *J Trauma*. In press.

4. Harro JM, Peters BM, O'May GA, Archer N, Kerns P, Prabhakara R, **Shirtliff ME**. Vaccine development in Staphylococcus aureus: taking the biofilm phenotype into consideration. *FEMS Immunol Med Microbiol*. 2010 Aug;59(3):306-23.

5. Hospenthal DR, Green AD, Crouch HK, English JF, Pool J, Yun HC, Murray CK, **Prevention of Combat-Related Infections Guideline Panel**. Infection prevention and control in deployed military medical treatment facilities. *J Trauma*. In press.

6. Hospenthal DR, Murray CK, Andersen RC, Bell RB, **Calhoun JH,** Cancio LC, et al. Executive summary: Guidelines for the prevention of infections associated with combat-related injuries: 2011 update. *J Trauma*. In press.

7. Hospenthal DR, Murray CK, Andersen RC, Bell RB, **Calhoun JH,** Cancio LC, et al. Guidelines for the prevention of infections associated with combat-related injuries: 2011 Update. *J Trauma*. In press.

8. Martin GJ, Dunne JR, Cho JM, Solomkin JS, **Prevention of Combat-Related Infections Guideline Panel**. Prevention of infections associated with combat-related thoracic and abdominal cavity injuries. *J Trauma*. In press.

9. Murray CK, Obremskey WT, Hsu JR, Andersen RC, **Calhoun JH**, Clasper JC, Whitman TJ, Curry TK, Fleming ME, Wenke JC, Ficke JR. Prevention of infections associated with combat-related extremity injuries. *J Trauma*. In press.

10. Peters BM, Jabra-Rizk MA, Scheper MA, Leid JG, Costerton JW, **Shirtliff ME**. Microbial interactions and differential protein expression in Staphylococcus aureus -Candida albicans dual-species biofilms. *FEMS Immunol Med Microbiol*. 2010 Aug;59(3):493-503.

11. Petersen K, Coyer MH, Hayes DK, Hale RG, Bell RB, **Prevention of Combat-Related Infections Guideline Panel**. Prevention of infections associated with combat-related eye, maxillofacial, and neck injuries. *J Trauma*. In press.

12. Prabhakara R, Harro JM, Leid JG, Harris M, **Shirtliff ME**. Murine immune response to a chronic Staphylococcus aureus biofilm infection. *Infect Immun*. 2011 Apr;79(4):1789-96.

Presentations:

1. **Calhoun JH**. Autogenous Bone Grafting and Bone Morphogenetic Proteins. Musculoskeletal Infection Society Annual Open Scientific Meeting, Los Angeles, CA, Aug. 6, 2010.

2. **Calhoun JH**. The Diabetic Foot. University of Louisville Bone and Joint Infection Center, Department of Orthopaedic Surgery and Podiatric Medicine, Division of Infectious Diseases, Forum on Diabetic Foot Infections, Louisville, KY, July 12, 2010.
3. **Shirtliff ME.** Advancements in the fight against biofilms in chronic infections. ATACCC 2010 Conference. St. Petes Beach, Florida. August 17, 2010.

4. **Shirtliff ME.** Biofilms and Biosafety. American Biological Safety Association 2010 Meeting. Denver, Colorado. October 2, 2010.

5. **Shirtliff ME.** Diagnosis and vaccine prevention of biofilm-mediated chronic orthopaedic infections. AOTrauma CPP Bone Infection workshop. Boston, Massachusetts. July 23, 2010.

6. **Shirtliff ME.** Novel vaccine strategies and improved diagnostics for musculoskeletal infections. AO Trauma eCM XII: Implant Infection. Davos, Switzerland. June 22-24, 2011.

7. **Shirtliff ME.** Staphylococcus aureus biofilm infection models. Eurobiofilms 2011. Copenhagen, Denmark. July 5, 2011.

8. **Shirtliff ME.** Who's smarter: The bugs or us? 2010 Orthopaedic Trauma Association. Baltimore, Maryland. October 16, 2010.

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Resolution of *Staphylococcus aureus* Biofilm Infection Using Vaccination and Antibiotic Treatment▽†

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Staphylococcus aureus **infections, particularly those from methicillin-resistant strains (i.e., MRSA), are reaching epidemic proportions, with no effective vaccine available. The vast number and transient expression of virulence factors in the infectious course of this pathogen have made the discovery of protective antigens particularly difficult. In addition, the divergent planktonic and biofilm modes of growth with their accompanying proteomic changes also demonstrate significant hindrances to vaccine development. In this study, a multicomponent vaccine was evaluated for its ability to clear a staphylococcal biofilm infection. Antigens (glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein) were chosen since they were found in previous studies to have upregulated and sustained expression in a biofilm, both *in vitro* and *in vivo*. Antibodies against these antigens were first used in microscopy studies to localize their expression in *in vitro* biofilms. Each of the four antigens showed heterogeneous production in various locations within the complex biofilm community in the biofilm. Based upon these studies, the four antigens were delivered simultaneously as a quadrivalent vaccine in order to compensate for this varied production. In addition, antibiotic treatment was also administered to clear the remaining nonattached planktonic cells since the vaccine antigens may have been biofilm specific. The results demonstrated that when vaccination was coupled with vancomycin treatment in a biofilm model of chronic osteomyelitis in rabbits, clinical and radiographic signs of infection significantly reduced by 67 and 82%, respectively, compared to infected animals that were either treated with vancomycin or left untreated. In contrast, vaccination alone resulted in a modest, and nonsignificant, decrease in clinical (34% reduction) and radiographic signs (9% reduction) of infection, compared to nonvaccinated animal groups untreated or treated with vancomycin. Lastly, MRSA biofilm infections were significantly cleared in 87.5% of vaccinated and antibiotic-treated animals, while antibiotics or vaccine alone could not significantly clear infection compared to controls (55.6, 22.2, and 33.3% clearance rates, respectively). This approach to vaccine development may lead to the generation of vaccines against other pathogenic biofilm bacteria.**

While once only a hospital-acquired pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA) infection has spread to the community and is now reaching epidemic proportions. A recent study has found that nearly 19,000 people per year die from MRSA infections in the United States, a death toll higher than that due to AIDS (16). In addition, up to 20% of patients who undergo surgery acquire at least one nosocomial infection (14), which is estimated to add $5 to $10 billion in costs to the U.S. healthcare system. *S. aureus* is one of the most common etiologic agents of these infections. These numbers of deaths, as well as the associated healthcare costs, do not even take into account the morbidity and mortality caused by methicillin-sensitive *S. aureus* (MSSA) strains that still cause the majority of staphylococcal infections. Therefore, the generation of a vaccine that is protective against *S. aureus* would have the potential to significantly reduce the morbidity and mortality associated with these infections. One of the major ways that *S. aureus* is able to persist is through growth as a biofilm, which is recalcitrant to clearance by antimicrobials, further limiting the efficacy of currently available antibiotics. With fewer appropriate means of treating the illnesses caused by this bacterium, the prevention of disease is essential.

There have been several approaches to designing an effective *S. aureus* vaccine. Whole live or killed *S. aureus* vaccines have proved to be largely ineffective in animal models (40). Thus, research has focused on using purified forms of either polysaccharide or protein from the bacterial surface. Much research has centered on the capsular polysaccharide types 5 and 8. One such vaccine, StaphVAX, demonstrated protective efficacy in animal models of infection; IgG produced as a result of vaccination showed high levels of opsonophagocytosis *in vitro* (10) and in a phase III clinical trial. However, protection waned with time and by 1 year postvaccination was <30% (34). Active or passive immunization with polysaccharide intracellular adhesin (PIA), the principal exopolysaccharide component of *S. aureus* and *S. epidermidis* biofilms, has been shown to

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† Supplemental material for this article may be found at http://iai.asm.org/.

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TABLE 1. Primers and plasmids utilized in this study

Primer or plasmid	Sequence (5′–3′) or genotype/characteristics[a]	Product or source
Primers		
5′SA0037	ATGAATACAATCAAAACTACGAAA	Conserved hypothetical protein (519 bp)
3′SA0037	CTTCTCATCGTCATCTGATTTCAAAATCCATTTTTGA	
5′Lipase	ACTCTAGGTCTCACTCCCATCTGAAACAACATTATGACCAAAT	Lipase (966 bp)
3′Lipase	ATGGTAGGTCTCATATCATAAAGGATTTAACGGTAATTCATTACT	
5′SA0688	ATGGTAGGTCTCACTCCGATAAGTCAAATGGCAAACTAAAAGT	ABC transporter lipoprotein (860 bp)
3′SA0688	ATGGTAGGTCTCATATCATTTCATGCTTCCGTGTACAGTT	
5′Glucosaminidase	ATGGTAGGTCTCACTCCGCTTATACTGTTACTAAACCACAAAC	Glucosaminidase (1,443 bp)
3′Glucosaminidase	ATGGTAGGTCTCATATCATTTATATTGTGGGATGTCGAAGTATT	
5′SA0486	ACTCTAGGTCTCACTCCAAAGAAGATTCAAAAGAAGAACAAAT	Hypothetical lipoprotein (683 bp)
3′SA0486	ATGGTAGGTCTCATATCAGCTATCTTCATCAGACGGCCCA	
Plasmids		
pBAD-Thio/TOPO	4,454 bp; pUC ori, Amp^r, pBAD promoter, for arabinose-inducible expression of PCR product	Invitrogen Life Technologies
pASK-IBA14	3,001 bp; pUC ori, Amp^r, tetA promoter, for tetracycline-inducible expression of PCR product	IBA, Göttingen, Germany

[a] BsaI sites are underlined in the primer sequences. Amp^r, ampicillin resistance.

be protective against *S. aureus* infection in a kidney infection model (25). However, recent research has illustrated that only one component of PIA is immunogenic, and responses to this antigen are variable (22). Deacetylation of PNAG, as well as conjugation to diphtheria toxin as a carrier protein, does help increase protection levels (23). However, not all clinical isolates of either *S. aureus* or *S. epidermidis* produce PIA (11, 27, 28, 31), making the relevance of such a vaccine questionable.

Protein-based vaccines have focused mainly on the microbial-surface-component-recognizing adhesive matrix molecules (MSCRAMM) subset of cell wall-associated proteins. Individual component vaccines consisting of clumping factor A (ClfA), ClfB, iron-regulated surface determinant B (IsdB), and fibronectin-binding protein (FnBBP) have all been tested. Recombinant ClfA was shown to be only partially protective when used in an animal model of septic arthritis (15). ClfA is also being investigated as a DNA vaccine candidate in mice and cattle. However, while injection of plasmid containing *clfA* increased clearance in a mastitis model, protection was not generated against infection in an intraperitoneal challenge (6). Immunization with rClfB led to lessened colonization of murine nares by *S. aureus* (33). Vaccination with IsdB led to increased survival rates of 20 to 40% in a murine sepsis model (18). A fusion protein consisting of the binding regions of Cna (collagen binding protein) and FnBP showed some protection in a mouse intraperitoneal model (41).

The vaccines discussed above have several limitations, including incomplete protection and the differential expression of the component proteins among *S. aureus* isolates (26, 32). Use of a multicomponent vaccine has shown promise in promoting significant protection against *S. aureus* infection. When IsdA and IsdB, as well as SdrD and SdrE, were combined into a single vaccine, complete protection was afforded in a mouse renal abscess model, with bacterial levels being reduced below levels of detection and a lack of clinical signs of disease (37).

Even with the advancements being made in this field, the vast majority of research focuses on protection from acute, plankton-associated *S. aureus* infection. Also, the studies discussed above all make use of non-biofilm animal models of infection. Because we (4) and others (2, 29) have shown that gene expression and protein production between the two states of biofilm and planktonic modes of growth differ greatly, the vaccine candidates that prevent infection in acute, plankton-associated models (for example, sepsis, intraperitoneal infection, and renal abscess models) may not be effective against biofilm infections such as osteomyelitis, endocarditis, or prosthetic implant infections.

Previous work in our laboratory identified several cell wall and membrane-associated proteins that are immunogenic during *S. aureus* biofilm infection and whose genes are upregulated during biofilm growth (4). In the present work, recombinant forms of four of these proteins were combined in a quadrivalent vaccine and tested for their ability to provide protection against challenge with an *S. aureus* biofilm infection that is normally recalcitrant to antimicrobial clearance. An antibiotic, while not effective against biofilm communities, was also used in conjunction with vaccination for the clearance of any remaining planktonic staphylococci. The quadrivalent vaccine with antibiotic therapy was effective in clearing the infection, while either vaccination or antibiotic treatment alone were not. This study is the first to acknowledge, and overcome, the differences of protein expression within biofilms and, as such, suggests a possible alternative in rational vaccine design for other biofilm-mediated infections.

MATERIALS AND METHODS

Bacterial strains. MRSA strain MRSA-M2 was isolated from a patient with osteomyelitis at the University of Texas Medical Branch. *Escherichia coli* TOP10 and BL21 cells were utilized for protein production experiments.

Cloning, expression, and purification of proteins. Candidate antigens selected in Brady et al. (4) were amplified using the primers listed in Table 1. The PCR products were cloned into pBAD-Thio/TOPO (SACOL0037) or pASK-IBA14 (SACOL0486, SACOL0688, and glucosaminidase), transformed into TOP10 *E. coli*, and sequenced. The clones were then expressed using either arabinose induction (SACOL0037) or anhydrotetracycline induction (all others). SACOL0037 was purified via nickel affinity chromatography, while all other antigens were purified by using Strep-Tactin Superflow columns (IBA, Göttin-

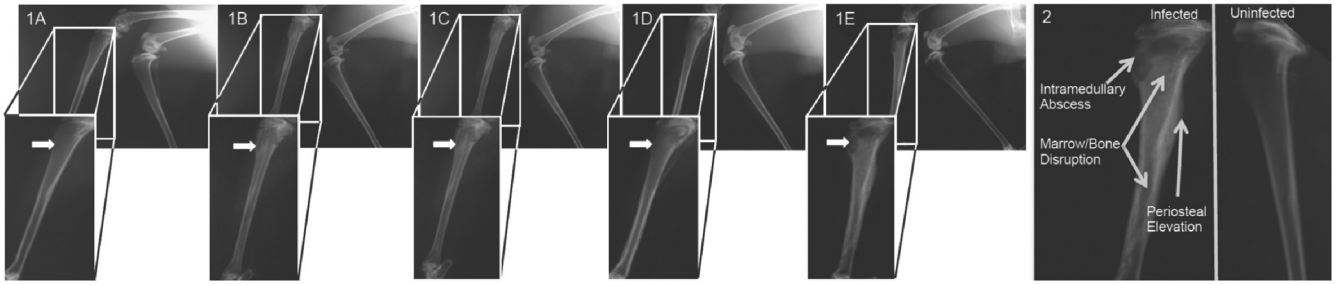

FIG. 1. Radiographic differences between infected and uninfected tibias. (Panels 1A to 1E) Left tibiae are shown exhibiting radiologic scores of 0 in a representative animal (1A), with increasing scores being shown to a maximum radiologic score of 4 in a representative control animal (1E) where arrows designate areas of *S. aureus* injection sites. In each panel, the right (noninoculated) tibiae are also shown and serve as internal controls for scoring. (Panel 2) Expanded view of a radiographic image of an infected (left) tibia demonstrating areas of abscess, marrow and bone disruption, and periosteal elevation in an infected tibia compared to an uninfected tibia (right). For complete descriptions of the scoring parameters, please refer to Table 2 and the supplemental materials and methods.

gen, Germany). The purity was confirmed by resolving each protein on SDS–15% PAGE, and quantities were determined by using BCA (Pierce, Rockford, IL).

Evaluation of antigen expression in *S. aureus* biofilms *in vitro*. Purified proteins were used to develop polyclonal antibodies through a commercial source (Lampire, Inc., Everett, PA). Antibodies were purified from the serum and used to probe 14-day-old *S. aureus* biofilms grown *in vitro* as described previously (4, 5).

Vaccination of animals. To prepare the purified recombinant proteins for vaccination, the appropriate amounts of SA0037, SA0486, SA0688, and glucosaminidase were combined. Because we noticed faint extraneous bands in our SA0037 preparation following purification on the Probond column, we took a further step of resolving rSA0037 using SDS-PAGE and cutting out the proper band. This band was then resuspended in 250 μl of phosphate-buffered saline (PBS), homogenized, and the mixture was used to rehydrate the trichloroacetic acid precipitation. The rehydrated protein was combined with an equal volume of Titermax Gold adjuvant (Titermax USA, Norcross, GA) and mixed via sonication.

Eight-week-old female New Zealand White rabbits (2-3 kg each) were used in the present study. All procedures were performed as per humane criteria set forth by University of Maryland Baltimore Animal Care and Use Committee. Animals were divided at random into groups. Groups received glucosaminidase, the quadrivalent vaccine, or PBS as a control. For the initial testing of glucosaminidase alone or the quadrivalent vaccine, animals were immunized with 10 μg of each antigen intramuscularly at days 0, 10, and 20, with challenge following on day 30. In all of the remaining other vaccine studies, animals were immunized with either 75 μg each of antigen intramuscularly, or the PBS control, at days 0 and 10. Antibody titers to vaccine antigens were measured by enzyme-linked immunosorbent assay (see supplemental materials and methods). Titers against antigens increased 10 days after vaccination and continued to rise until day 35 (see supplemental material and Table S1).

Production of osteomyelitis. Animals were challenged 10 days following the last vaccination with intratibial inoculation of MRSA-M2 as described previously (21). All procedures were performed as per humane criteria set forth by University of Maryland Baltimore Animal Care and Use Committee. The infection was allowed to progress for 14 days, at which time the animals were evaluated and euthanized for the first study with glucosaminidase alone and the quadrivalent vaccine. In the subsequent study for testing the adjunctive effects of antimicrobial therapy, animals were either left alone or treated for 14 days via a twice daily subcutaneous injection of vancomycin (40 mg/kg) as previously described (20).

Analysis of vaccine efficacy. Vaccine efficacy was evaluated in three ways. At 14 days following MRSA inoculation into the tibia, rabbits were monitored for clinical signs of infection (i.e., non-weight bearing on the affected leg). Animals were then anesthetized and radiographically examined to determine the radiologic score of osteomyelitis, according to Fig. 1 and described previously (21). Scores were evaluated as shown in Table 2. Rabbits were then sacrificed by an intravenous injection of sodium pentobarbital. Both tibias were removed, dissected free of all soft tissue, and processed for bacterial cultures. Using a 5.0-mm, single-action rongeur, the bones were split into small pieces, and the marrow was removed. The whole bone was then pulverized, combined with the marrow, and suspended in 3 ml of sterile 0.85% saline per g of tissue. Tenfold serial dilutions were performed in triplicate and spotted onto a tryptic soy agar blood plate supplemented with oxacillin (40 μg/ml) to determine the presence or absence of *S. aureus* in the bone tissue. The CFU per gram of bone were then calculated after overnight incubation of the plates at 37°C. For more details concerning rabbit tibial culture, refer to the supplemental materials and methods.

Statistical analysis. The statistical significance between experimental groups and controls, as well as between the various experimental groups, was calculated by using a Student *t* test for radiologic and CFU data and the Fisher exact test for clinical symptoms and infection clearance rates. A P value of ≤ 0.05 was considered statistically significant, while P values between 0.1 and 0.005 were considered to show a trend.

RESULTS

Biofilm upregulated immunogens are produced heterogeneously within the biofilm. Differential protein production within the biofilm may allow certain portions of the biofilm to escape immune recognition and clearance. Therefore, we chose to study a subset of candidate antigens. We used IgG samples from animals vaccinated against individual antigens, with confocal immunofluorescence microscopy on *S. aureus* biofilms grown *in vitro*, to determine the relative areas of production a number of other biofilm upregulated antigens, glucosaminidase, SA0317 (lipase), SA0486 (a hypothetical lipoprotein), SA0037 (a conserved hypothetical protein of unknown function), SA0688 (an ABC transporter lipoprotein), and SA. As seen in previous studies (5), there was heterogeneous production of proteins within the biofilm community (Fig. 2). IgG against each antigen (with the exception of lipase) appear to bind to *S. aureus* biofilms differently. Although anti-glucosaminidase and anti-SA0688 IgGs bind to individual microcolonies, anti-SA0486 IgG reacts with smaller bacterial flocs within the entire biofilm, and anti-SA0037 IgG binds to individual cells within microcolonies. In addition, lipase, which

TABLE 2. Radiographic staging guidelines

Radiological score	Characteristics of infected bone
0	Normal, no lytic changes around needle stick
1+	Lytic changes around the needle stick, <5% disruption of normal bone architecture
2+	5–15% disruption of normal bone architecture
3+	15–40% disruption of normal bone architecture
4+	>40% disruption of normal bone architecture

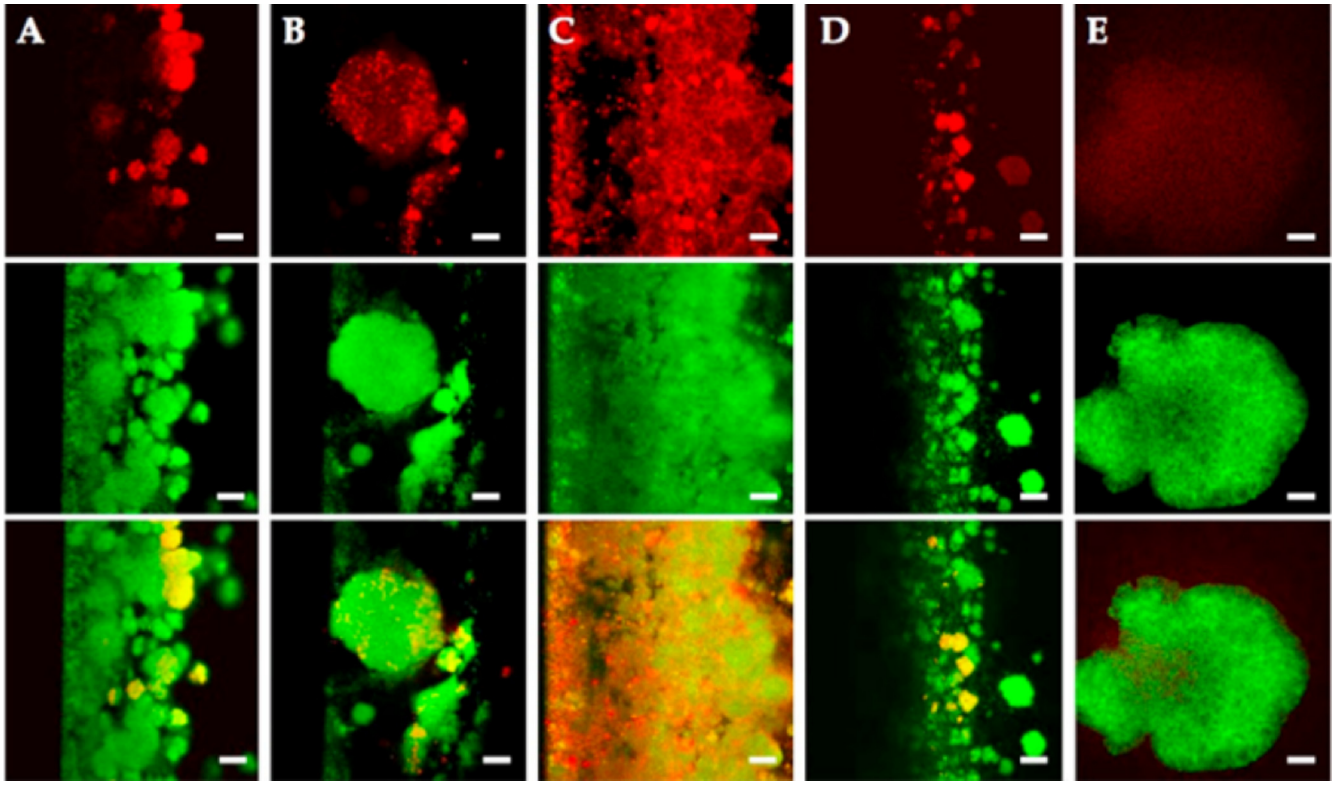

FIG. 2. Biofilm-upregulated immunogens are produced heterogeneously. Immunofluorescence microscopy was performed using IgG antibodies against biofilm upregulated immunogens, followed by goat anti-rabbit F(ab′)$_2$ secondary antibody (red, top panels) and SYTO-9 stain to visualize the entire biofilm (green, center panels; merge, bottom panels). (A) Glucosaminidase; (B) SA0037; (C) SA0486; (D) SA0688; (E) lipase, a secreted protein not found in large quantities within the biofilm (negative control). Magnification bars, 20 μm.

is secreted by *S. aureus* in a biofilm mode of growth, serves as a negative control. Because this enzyme is produced and released into the flowing media, IgGs against lipase did not bind to the biofilm. Thus, it was evident that these antigens are not expressed homogeneously throughout the biofilm and that antibodies to a single antigen may not provide adequate immunological recognition of the biofilm, leaving some areas that were not recognized and thus persist. Therefore, we further tested a single component and quadrivalent vaccine in subsequent studies (see below).

Vaccination with a single biofilm antigen or quadrivalent vaccine. In previous work we identified 22 cell wall and membrane-associated immunogens that were upregulated during biofilm growth (4). Among these antigens, autolysin (AtlA) was one of the most immunoreactive. Because of its reported role in biofilm formation (3) and its upregulation during early biofilm growth (when an immune response could theoretically eradicate the biofilm), we chose to test a component of autolysin alone and a combined quadrivalent set of biofilm upregulated antigens as potential vaccines. The quadrivalent vaccine included SA0486, SA0037, SA0688, and glucosaminidase. We chose these antigens because we showed in earlier work (4) that they are cell wall associated, biofilm upregulated, and immunogenic in rabbits. Purified recombinant glucosaminidase (one of the two protein components of AtlA) or the quadrivalent vaccine was injected into rabbits (two doses of 10 μg of each antigen, 10 days apart), and then animals were challenged using a tibial osteomyelitis infection. This vaccination did not lead to significant differences in bacteriological signs of infection compared to PBS-vaccinated controls but did yield significantly lower radiological scores (Table 3 and Fig. 3).

The failure of the single and quadrivalent vaccine alone to promote effective bacterial clearance may have been due to the inability of the immune system to clear planktonic cells, since the antigen was a biofilm upregulated protein. Although the single antigen (i.e., glucosaminidase) and the quadrivalent vaccine showed similar clinical and radiological reductions in infection, we did not test this single antigen in subsequent studies for a number of reasons. First, there have been a number of generated, as well as naturally spontaneous, mutants arising in this particular gene (12, 13, 38). Therefore, a vaccine com-

TABLE 3. Radiological and clinical vaccine scores for glucosaminidase and quadrivalent vaccines

Parameter	Glucosaminidase	Quadrivalent	PBS control
No. of rabbits	4	5	7
Mean radiological score[a]	0.37*	1.10*	2.71
Rabbits (%) showing clinical signs of infection	0	0	57
No. of rabbits cleared	1	0	0

[a] *, $P < 0.05$ glucosaminidase versus PBS control and quadrivalent versus PBS control.

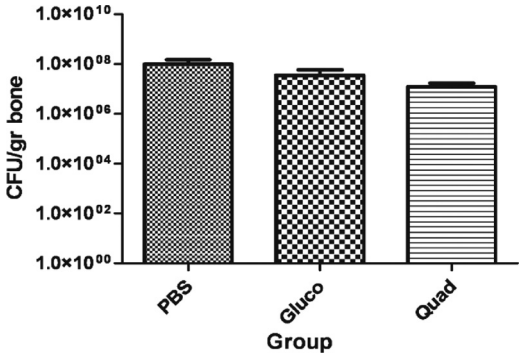

FIG. 3. Vaccination with the single antigen glucosaminidase and the quadrivalent vaccine. The mean CFU/gram of bone are shown for PBS controls, glucosaminidase-vaccinated (Gluco) and quadrivalent-vaccinated groups. Animals were vaccinated with three doses of the quadrivalent vaccine (10 μg of each) or PBS.

posed of this single component may be short-lived in usefulness due to null mutant infecting strains that could escape clearance by the immune response. Also, the well-described heterogeneous antigenic nature of biofilms in our studies and many others supports the idea that a multicomponent vaccine may be needed for protection against biofilm-mediated disease (1, 5, 36). Lastly, a multivalent vaccine strategy is a standard method in many currently approved vaccines, including the pneumococcus and the acellular pertussis component of the DTaP vaccine (1, 17, 19).

Vaccination with biofilm-upregulated antigens, and subsequent antibiotic therapy, leads to clearance of biofilm infection. The ultimate goal of complete bacterial clearance was not realized with the single antigen and quadrivalent vaccine. Because there was an obvious, albeit statistically insignificant trend to reduced infection upon challenge with the quadrivalent vaccine, we hypothesized that vaccination with these biofilm upregulated antigens may reduce the number of bacterial cells with a biofilm phenotype. As a result, the bacterial populations remaining in the vaccinated group postchallenge may be due to the planktonic subset of *S. aureus* within the tibia not being effectively cleared by the host immune response. Planktonic *S. aureus* cells possess a number of immuno-avoidance strategies, including protein A, leukotoxins, an antiphagocytic capsule, and the recently described phenol soluble modulins and nitric oxide-inducible lactate dehydrogenase system (30, 39) that enable persistence. However, they are sensitive to effective antimicrobial agents compared to their biofilm-embedded counterparts. To this end, at 14 days after challenge we treated both vaccinated and nonvaccinated animals with 40 mg of vancomycin/kg twice daily for 10 days and compared the efficacy of the dual treatment to untreated and unvaccinated, vaccinated but untreated, and unvaccinated but treated controls. As in the studies described above, the vaccine alone group showed no significant effect on infection clearance or concentrations of bacteria in the tibia (Fig. 4B). However, both the vancomycin alone group and the group treated with vancomycin therapy following vaccination with the quadrivalent vaccine were able to significantly reduce bacterial counts in the affected tibia ($P < 0.05$) (Table 4 and Fig. 4). When clearance rates were observed, vaccination and antibiotic treatment afforded only this group significantly lower rates, as well as scores for clinical and radiological signs of disease ($P < 0.05$) (Table 4 and Fig. 4). Thus, this combination therapy is able to abrogate all signs of *S. aureus* osteomyelitis infection and, in the rare case clearance is not achieved, bacterial levels (as well as severity of disease) are markedly decreased. Therefore, the combined use of a prophylactic, biofilm-directed vaccine, plus antibiotic treatment aimed at planktonic growth, leads to the prevention of biofilm-mediated osteomyelitis infection in a rabbit model.

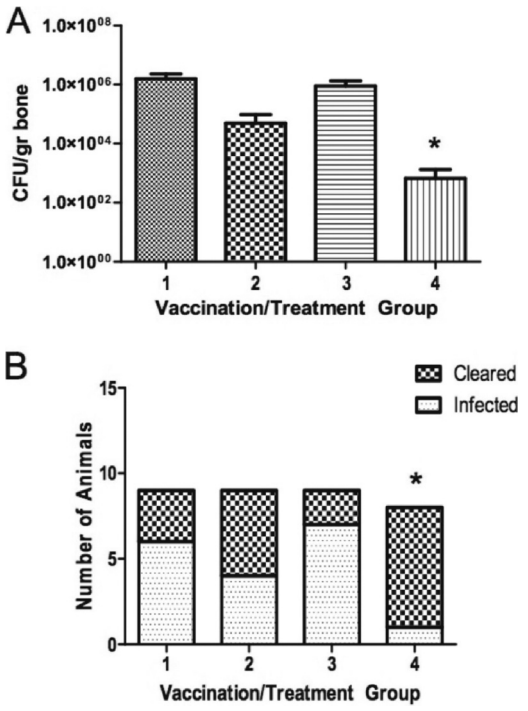

FIG. 4. Vaccination with quadrivalent vaccine and adjunctive vancomycin treatment. (A) Animals vaccinated with PBS only (column 1), PBS and subsequent treatment with vancomycin (column 2), the quadrivalent vaccine only (column 3), or the vaccine plus vancomycin (column 4). The mean ± the standard error of the mean for CFU/gram of bone is shown for each group. *, Significant difference from group 1, the PBS control ($P < 0.05$, Student *t* test). (B) Animals in each group that were completely cleared of infection. *, Significant difference from group 1, the PBS control ($P < 0.05$, Fisher exact test).

TABLE 4. Radiological and clinical scores for quadrivalent vaccine with vancomycin therapy

Parameter	Group 1 (PBS)	Group 2 (PBS + vancomycin)	Group 3 (vaccine)	Group 4[a] (vaccine + vancomycin)
No. of rabbits	9	9	9	8
Mean radiological score	2.3	2.1	2.0	0.4*
Rabbits (%) with clinical signs of infection	100	100	66	38*
No. of rabbits cleared	3	5	2	7*

[a] *, $P < 0.05$ of vaccine plus vancomycin group 4 versus PBS control group 1.

DISCUSSION

S. aureus has re-emerged as a major human pathogen, and there are currently no vaccines that afford consistent, long-term protection against *S. aureus* infections. While infections, particularly those with MRSA, are often nosocomial in origin, community acquired infections associated with this microbial species have reached epidemic levels. One of the ways in which *S. aureus* is able to persist in the host and remain recalcitrant to clearance by the immune system or antibiotics is through a biofilm mode of growth. Therefore, the need for an effective vaccine and/or treatment modality that could prevent the establishment of biofilm-mediated chronic infections by *S. aureus* is necessary. This study is the first to demonstrate protection against biofilm-associated infection through the use of a multicomponent vaccine and subsequent antibiotic therapy. When administered to New Zealand White rabbits, the combination of biofilm-directed vaccination and antibiotic treatment was able to significantly lessen the radiological and clinical signs of infection, and afforded complete clearance to 87.5% of animals, reducing bacterial loads overall by over 3 logs.

A number of vaccines have been evaluated for their protective efficacy against staphylococcal infections, in particular against a primary planktonic bacteremia, pneumonia, septic arthritis, and intraperitoneal staphylococcal infection (6, 15, 18, 24, 25, 34, 37). Although this mode of growth is important and can often end in septicemia and death, it can be cleared by antimicrobial therapy and may be the transient intermediary step between inoculation and rapid dissemination to distal sites of biofilm infection. Therefore, a long-term T-cell-mediated memory response and antibody production against staphylococcal antigens for this transient and antibiotic sensitive bacteremia may take up to 10 days for full activation. This may not be rapid enough to clear the infection prior to the development of a secondary biofilm infection that can occur within several days postinoculation and will resist clearance by antimicrobial agents (35).

Because of the complicated multicellular architecture of a biofilm, various sites within these communities can, and do, express different proteins necessary for survival under various respiratory conditions and areas of stress (7–9). When we tested our single antigen and quadrivalent vaccine in our chronic osteomyelitis model, we noted that both the clinical and radiological signs of disease were significantly decreased in the vaccinated group compared to controls; however, a lessened, but nonsignificant, number of bacteria were still found in the bones of vaccinated animals. This suggests that the vaccine is working against biofilm bacteria and decreasing the manifestations of osteomyelitis but is not clearing out all of the *S. aureus* present in the bone. Previous work by our laboratory demonstrated that antibodies against biofilm-upregulated proteins could be used to study biofilm architecture *in vitro* (5); here, we expanded on this work to more closely examine the expression states of our potential vaccine candidates within an intact biofilm. We noted that there is heterogeneous expression of each of the four candidate antigens in a mature biofilm. This could lead to vaccine-based selection of those areas of the biofilm that do not express the candidate antigen to persist and spread infection. Other vaccine studies (37) have suggested a multicomponent vaccine may afford more complete protection against *S. aureus* challenge in a planktonic model of infection.

A mature biofilm is recalcitrant to clearance by both the host immune response and antimicrobial therapies (7–9). Therefore, use of antibiotics against a biofilm infection is generally not effective. The benefit of a biofilm-directed vaccine would be to generate a memory response that can be elicited quickly upon challenge with the etiologic organism, generating a protective response that can work against early biofilm microbes that are still in a clearable state. Because the antigens chosen for this vaccine were those that were significantly upregulated during biofilm growth, we postulated that the vaccine was not effective against planktonic bacteria and, thus, the response elicited through vaccination and subsequent challenge may not have been sufficient to eradicate all populations. We therefore added an antibiotic treatment arm to our study, comparing the effectiveness of prophylactic vaccination combined with post-challenge vancomycin therapy to vaccination alone, and also of vancomycin treatment without prior vaccination. While vaccination alone was not able to significantly decrease the levels of *S. aureus* in the infected bone, both vancomycin treatment alone and the combination of vaccination and antibiotic therapy significantly reduced *S. aureus* numbers. Although reductions in bacterial populations are important, significantly increased infection clearance rate are essential for a potential treatment or prevention strategy since any remaining bacteria can regrow to produce a fulminant infection. When clearance rates were compared, only the vaccination combined with antibiotic treatment was able to significantly eliminate the *S. aureus* infection from the host.

This vaccine holds significant promise for those with identified risk factors for *S. aureus* biofilm infection. Although these patients may still acquire the *S. aureus* infection, an anti-biofilm vaccine could allow these previously untreatable infections to be prevented by vaccination in combination with antimicrobial therapy, whereas the only reliable therapy at present is surgical intervention. These data give new perspectives on means to limit and eradiate *S. aureus* biofilm infections that could help to prevent the onset of chronic disease, saving patients from significant morbidity and mortality. As well, the methodology used here, where the entire microbial community is considered for its *in vivo* expression and differential protein production in the various niches of the biofilm, has implications for how future biofilm vaccines should be designed. This suggests a potential alternative in how antigens are rationally chosen for these infections.

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Prevention of Infections Associated With Combat-Related Burn Injuries

AQ: 4

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Abstract: Burns are a very real component of combat-related injuries, and infections are the leading cause of mortality in burn casualties. The prevention of infection in the burn casualty transitioning from the battlefield to definitive care provided at the burn center is critical in reducing overall morbidity and mortality. This review highlights evidence-based medicine recommendations using military and civilian data to provide the most comprehensive, up-to-date management strategies for initial care of burned combat casualties. Areas of emphasis include antimicrobial prophylaxis, debridement of devitalized tissue, topical antimicrobial therapy, and optimal time to wound coverage. This evidence-based medicine review was produced to support the *Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update* contained in this supplement of *Journal of Trauma*.

Key Words: Burns, Thermal injury, Military, Combat, Infection.

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Thermal injury is common to all modern military conflicts.[1] As a result of explosive devices being used against military personnel involved in Operation Iraqi Freedom and Operation Enduring Freedom, burns were identified as the primary cause of injury in ~5% of military personnel evacuated from these battlefields.[2] The concept of the dedicated burn unit is a product of wartime and disaster experience and is closely tied to developments in infectious disease treatment. Archibald McIndoe, civilian consultant to the Royal Air Force in plastic surgery, established a burn ward at the East Grinstead hospital in 1940. The focus of his work was postburn reconstruction.[3] After the Cocoanut Grove nightclub fire in Boston in 1942, Cope et al. established a temporary ward at the Massachusetts General Hospital dedicated exclusively to the care of the surviving burn patients. The results of the Cocoanut Grove experience were carefully documented in a monograph with the chapter on infections written by Dr. Champ Lyons, a surgeon and microbiologist.[4] Lyons later became the director of the Wound Unit at Halloran General Hospital, Staten Island, NY, the forerunner of the US Army Surgical Research Unit.[5] The initial focus of the unit was to characterize the role of newly discovered antibiotics in the treatment of war wounds.[6] The Surgical Research Unit moved to Fort Sam Houston, TX, in 1947, and the US Army Burn Center was established there in 1949, in response to the growing threat of nuclear war and concern that the large number of burn injuries that resulted from the bombing of Hiroshima would characterize future conflicts.[7] Once established, the US Army Burn Center focused research efforts on improving postburn resuscitation and preventing renal failure and burn wound sepsis.[7] The research in these areas has continued to evolve with ensuing military conflicts.

The evacuation of burned personnel has also evolved with each new conflict to which the US military has responded. During the Vietnam War, burned personnel were evacuated to an US Army general hospital in Japan, where they were treated for variable periods (days to weeks) before transfer to the United States.[1,8,9] During the operations in Iraq and Afghanistan, thermally injured US military personnel have arrived in the continental US (CONUS) for definitive care ~4 days after injury.[10] During the course of an evacuation from Iraq or Afghanistan, patients transition through several medical facilities with differing levels of capabilities before arriving at a major US medical center.

The US military currently uses a role-based treatment and evacuation in which injured personnel initially receive basic resuscitation and hemorrhage control by embedded military medics (role 1). Some patients undergo initial medical therapy at facilities staffed by physicians or physician assistants (role 2a). Casualties who require further care are transported to a facility that can provide initial surgical intervention, such as a forward surgical team (role 2b), or more often a combat support hospital (role 3) that contains surgical subspecialists and intensive care capabilities. Personnel who require ongoing care are transported to Landstuhl Regional Medical Center in Germany (role 4) and from there, burn casualties are transported to the US Army Institute of Surgical Research (USAISR), the US Army Burn Center at

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Fort Sam Houston, TX (role 5). The method of transport varies with the severity of injury. The most critically injured patients are transported by the USAISR Army Burn Flight Team or US Air Force Critical Care Air Transport Teams. Burn casualties with less severe burns and ambulatory patients with minimal injuries may be transported on scheduled evacuation flights supported by US Air Force Aeromedical Evacuation teams.[11] The criteria for evacuation of burn patients from theater based on burn severity are listed in Table 1. Evacuation to the USAISR is recommended for casualties with moderate or severe burns or any burns involving the hands, face, or perineum. In addition to surgical and nursing expertise, the USAISR provides the intensive rehabilitation and psychologic support necessary for these patients throughout the recovery process, as well as future reconstructive surgery.

Historically, burn wound infection was the most common cause of death in the thermally injured patient. Fortunately, advances in care have led to a decline in the occurrence of burn wound infection. However, wound infection remains a concern, particularly in the setting of delays in definitive surgical care, such as may occur in the combat environment. A recent autopsy study of 74 burns patients treated at the USAISR Burn Center found infection of wounds or the lower respiratory tract were the causes of death in 61% of patients.[12] The 36 patients who sustained burn injuries as a result of combat operations in Iraq and Afghanistan were more likely to die from infection (75%) than the 38 patients who sustained noncombat-related burns (47%). The potential explanations for this finding are myriad but include differences in time to definitive care, differences in total body surface area (TBSA) burned, and differences in rates of inhalational injury between combat and noncombat burns. The clinical picture is further complicated by the fact that combat-associated burn casualties often suffer concomitant traumatic injuries. An evaluation of 540 combat-related burn casualties found that 50.9% had multiple traumatic injuries.[11] The best method of caring for thermally injured casualties, including those with multiple, concomitant traumatic injuries as they transition from the battlefield setting has yet to be determined. However, the importance of infection as a cause of mortality in this patient population cannot be overemphasized; therefore, the prevention of infections in the burn patient as he or she transitions from the battlefield to definitive care at USAISR is the focus of this review.

METHODS

A MEDLINE search was performed on December 15, 2010, and January 20, 2011, using the key words "burns," "thermal injury," "military," "combat," "infection," "prevention," and "wound infection."

Microbiology and Epidemiology of Burn Wound Infections

The microbial epidemiology of burn wound infections has evolved during the past 20 years as use of topical antimicrobials, routine wound care, early burn wound excision, and definitive coverage with autograft have become standard practices. Evidence suggests that the incidence of bacterial burn wound infection has declined, first because of effective topical antimicrobials and second because of the practice of early excision and grafting (although data on this latter practice are inconclusive in the setting of large burns).[13–17] A meta-analysis of all available randomized controlled studies found a reduction in mortality with early excision for all burn patients without inhalation injuries.[17] Early excision and grafting has become standard practice in most US burn centers. Early excision and grafting, before arrival at the USAISR Burn Center, is not currently practiced because it would further expose the patient's open wounds to the environment as they transit multiple facilities, across thousands of miles, enroute to definitive care.[11] Knowledge of pre-excision burn wound flora is important to understanding the risks for burn wound infection in military personnel.

Most of the available data on the bacteriology of burn wound infections have been taken from studies performed before the practice of early excision and grafting. Although the incidence of infection has decreased as a result of early excision and grafting, the list of offending microorganisms responsible for infection has not changed significantly.[12,18–22] In the absence of topical antimicrobials, the immediate postburn period is characterized by rapid colonization of the

TABLE 1. Recommendations for the Evacuation of Burn Patient From the Combat Zone[10]

Category	Burn Severity*	Evacuation Recommendation
1	Limited partial-thickness burns not involving hands, joint, face, and perineum	Air evacuation to Landstuhl for wound care with expected return to duty
2	Limited partial-thickness burns involving hands, joint, face, and perineum, or any limited full-thickness burn	Air evacuation to US Army Institute of Surgical Research (USAISR) Burn Center
3	Moderate partial- or full-thickness burns, patient stable	Transfer to USAISR Burn Center via Critical Care Air Transport Team (CCATT)
4	Severe partial- or full-thickness burns and/or inhalation injury requiring intubation, patient stable	Transfer to USAISR Burn Center via Burn Flight Team (Special Medical Augmentation Response Team, SMART-Burn)
5	Severe partial- or full-thickness burns, patient unstable for air evacuation to United States	Transfer to a European burn center
6	Vesicant casualties	Air evacuation to USAISR Burn Center

* Burn severity definitions: limited, <10% TBSA; moderate, 10% to 30% TBSA; severe, >30% TBSA.

injured tissue by resident microbial flora.[19–22] Gram-positive skin flora such as *Streptococcus pyogenes* and *Staphylococcus aureus* reside deep within skin appendages and colonize the wound within the first 24 hours to 48 hours after injury.[19,20] Endogenous gram-negative bacteria from the patients' gastrointestinal tracts, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Escherichia coli*, colonize the wound within the first 48 hours to 72 hours after injury.[19,20] Microorganisms may also be transferred to the burn wound from contaminated surfaces, equipment, or the hands of health care workers.[23–26] Of the many bacterial microorganisms that colonize the burn wound surface after injury, *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* are the most likely to result in an invasive infection.[12,18,21,28] This finding is in part a result of the array of virulence factors possessed by these organisms. An autopsy study of patients with burns sustained in combat operations in Iraq and Afghanistan identified *P. aeruginosa* and *K. pneumoniae* as the microorganisms most frequently associated with mortality.[12] A retrospective study performed on patients with combat-related burns admitted to the USAISR Burn Unit found *K. pneumonia* bacteremia to have a higher associated mortality then bacteremia caused by *P. aeruginosa* or *S. aureus*.[28] The increased mortality associated with *K. pneumoniae* bacteremia was independent of age and TBSA, which are the characteristics that historically have had the greatest impact on mortality for patients with burns. The mortality associated with this pathogen coupled with dwindling antibiotic treatment options because of its increasing rates of extended spectrum beta lactamase production highlight the importance of preventing invasive infection.

In addition to these pathogens, the US military health care system has experienced an increased rate of multidrug-resistant *Acinetobacter calcoaceticus-baumannii* (Acb) complex infections in military personnel injured in Iraq and Afghanistan. The UK military has also experienced an increase in Acb infections. A study performed in the United Kingdom by Miranda et al.[29] evaluated the microorganisms involved in wound colonization and infection in both plastic surgery and burn patients. The authors found that military patients with combat-associated injuries were more likely to have wound colonization or infection with *S. aureus*, Acb, and *P. aeruginosa* than civilians treated in the same center. However, the impact of Acb infections remains uncertain. A retrospective cohort study by Albrecht et al.[30] found that although multidrug-resistant Acb is a frequent cause of infection in burn patients, it did not independently affect mortality in this population.

Burn patients are also subject to tetanus if inadequately immunized. A minor burn wound has been associated with fatal tetanus in at least one case report.[31] Therefore, we strongly recommend that the tetanus immunization status of all burn patients be determined. Clinicians should administer tetanus immunization to patients whose last booster was given more than 5 years ago and tetanus vaccination plus antitetanus immunoglobulin should be administered to patients who have no history of vaccination. Booster vaccination should be administered at 4 weeks and 6 months for this latter group.

Yeasts (e.g., *Candida* spp.) and filamentous fungi (e.g., *Aspergillus* spp.) are of increasing importance as a cause of invasive burn wound infection since the introduction of topical antimicrobial agents that have diminished the impact of bacterial infection.[18,22] Candidal colonization of burn wounds is more common than invasive disease and may arise from an endogenous or exogenous source.[31–33] The filamentous fungi are uniformly acquired from an exogenous environmental source and are much more likely to cause invasive disease than *Candida* species.[32–36] The filamentous fungi commonly associated with burn wound sepsis include *Aspergillus* spp., *Fusarium* spp., and members of the Mucorales order of the Zygomycetes.[37] An autopsy study of patients with burns sustained in combat operations in Iraq and Afghanistan found organisms with *Aspergillus*-like morphology or *Mucor*-like morphologies to be the leading cause of mortality as a result of fungal infections.[12] In addition, these organisms were the next most common cause of infection-related mortality after *P. aeruginosa* and *K. penumoniae*. There have also been case reports of invasive wound infection caused by a variety of dematiaceous fungi such as *Curvularia* spp.[38] Infections caused by filamentous and dematiaceous fungi are clinically challenging as they prove difficult to diagnose in the absence of a biopsy with interpretation by a skilled pathologist. A recent retrospective analysis of patients with thermal burns admitted to the USAISR Burn Center found that fungal burn wound infection is an independent predictor of mortality in patients with TBSA of 30% to 60%.[33] Fungal pathogens typically become a concern later in the treatment course after patients have undergone operation and received broad spectrum antibacterials and should not be a frequent cause of infection in the first few days after injury.[22,32]

Viral infections of burn wounds are rarely reported but do occur. Members of the herpes virus family, including herpes simplex virus and varicella zoster virus, are the most common culprits.[36,39] Cutaneous disease typically occurs in healing partial thickness burns and donor sites.[39] Cutaneous infection follows a benign course if recognized and treated early with topical therapy. Fortunately, invasive disseminated herpes simplex virus or varicella zoster virus is a rare occurrence in the burn patient but should be considered in the patient with cutaneous disease, concomitant pneumonitis, hepatitis, or meningitis as these patients will require systemic therapy.[36,39]

Systemic Predebridement Antibiotic Prophylaxis

The use of prophylactic systemic antibiotics is now well accepted in a wide variety of settings, including the combat casualty who presents with traumatic injuries. However, for the treatment of burns, use of systemic antibiotics for prophylaxis of subsequent burn wound infection has not been proven effective, either routinely (e.g., on admission) or at the time of wound debridement. Note that debridement refers to the practice of removing devitalized tissue and debris in conjunction with routine wound care and dressing changes and should be distinguished from the surgical excision of the eschar. The early use of antibiotics such as

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penicillin or erythromycin (aimed at controlling *Streptococcus* outbreaks) has been anecdotally observed to be associated with an increase in infections caused by resistant *Staphylococcus*,[40] although this is not a uniform finding.[41] No study has demonstrated a reduction in burn wound infections with the use of prophylactic antibiotics, and at least one study has shown an increased incidence of infections from Gram negatives, including *Pseudomonas*.[42] The only exception to this finding might be the use of antibiotic prophylaxis against staphylococcal toxic shock (STS), which can be a problem in pediatric burn care.[43] However, the use of prophylactic antibiotics for prevention of STS in children remains controversial. Routine systemic antimicrobial prophylaxis is not recommended for the burned patient undergoing rapid evacuation for definitive care. There are insufficient data to recommend for or against its use in patients with concomitant inhalation injury, and insufficient data to recommend for or against its use in children. In the event that a burn patient suffers from concomitant traumatic penetrating injury or fracture, antibiotic prophylaxis should be administered in accordance with the updated clinical practice guidelines published in this *Journal of Trauma* supplement.

Systemic Perioperative Antibiotic Prophylaxis

Antibiotic prophylaxis has also been examined in burn surgery. Few studies have supported the use of systemic antibiotic prophylaxis during excision and grafting procedures. In particular, antibiotics appear to be of no value in the prophylaxis of wound infections accompanying surgery for small burns.[44] The role of perioperative prophylaxis for excision and grafting of large burns (>40% TBSA) has not been well studied. Early studies documented a significant incidence of transient bacteremia associated with wound manipulation,[45] but a more recent evaluation showed this incidence to be much reduced.[46] Antibiotic administration has been found to reduce the incidence of this transient bacteremia but not to affect outcomes.[47] A recently published study by Ramos et al.[48] found that the use of systemic antibiotics administered perioperatively to patients undergoing grafting of deep burns was associated with improved autograft survival. However, the study had several limitations, including a small sample size, and more extensive follow-up studies will be required. Because of the limited evidence, controversy on this topic exists; and burn units vary widely in their practices of providing perioperative antibiotic prophylaxis.[49,50] Although the data are inconclusive, the clinician may consider the use of perioperative systemic antibiotics for excision and grafting procedures. The ideal regimen will vary based on the local antibiotic resistance patterns. The current practice at the USAISR Burn Center is to provide 24 hours of perioperative antibiotic prophylaxis with vancomycin and amikacin.

It is crucial to note that systemic antibiotic therapy is clearly indicated in the surgical treatment of infected burn wounds. Empiric treatment of patients with large open wounds and evidence of infection may be necessary. Many patients with large burns develop symptoms such as fever and leukocytosis as a consequence of the systemic response to injury, rather than infection, further complicating decisions regarding the use of antibiotics.[51] Thus, diagnosis of burn wound infection requires close attention to the patient's overall clinical status and to daily inspection of the appearance of the wound, as described elsewhere.[52] Examination of full-thickness wound biopsies by a qualified pathologist is the definitive diagnostic procedure.[52]

Topical Antimicrobial Use

In contrast to the uncertainty regarding the use of systemic antibiotic prophylaxis for burns, the use of topical antimicrobials, in conjunction with aggressive wound care and early excision and grafting, has been associated with a significant decline in the incidence of burn wound infections.[17,52–54] Topical antimicrobials and aggressive wound care should be performed at the lowest role possible and should be continued as the patient moves through the subsequent roles of care. Aggressive debridement of debris and devitalized tissue may not be feasible at lower roles. In this situation, clean, dry dressings should be applied to burn wounds and topical antimicrobials may be withheld until the patient is transferred to a higher level of care. There are limited data on how soon after injury debridement and application of topical antimicrobials should be performed; the opinion of the authors is that this should be performed within 8 hours of injury, assuming concomitant traumatic injuries have been adequately addressed.

Management recommendations based on burn severity are summarized in Table 2. First-degree and superficial partial-thickness burns may be treated with topical antimicrobials and daily dressing changes alone.[52–54] The use of temporary biosynthetic materials such as Biobrane (UDL Laboratories, Rockford, IL) is also an option for superficial partial-thickness burns. There are no data related to use of Biobrane in a field or combat environment. However, it is strongly recommended that Biobrane be considered only for patients with clean, fresh burns, which are rarely encountered in the deployed environment.[55–57] We recommend that deep partial-thickness and full-thickness burns be treated with topical antimicrobials with twice daily dressing changes, followed by early excision and grafting at the burn center.[13–17,52–54] If definitive surgical care must be accomplished in theater, such as when evacuation of host nation patients is not possible, we recommend that the procedure be performed only at a role 3 facility to offer the benefits of staffing, supplies, and equipment related to this level of care.

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TABLE 2. Management of Burn Wounds Based on Depth[16,17,20,52–55,58,59]

Wound	Interventions
First degree	Symptomatic care
Superficial partial thickness	Topical antibiotics with twice-daily dressing change, silver-impregnated dressing changed every 3–5 d, or Biobrane*
Deep partial thickness	Topical antibiotics with twice-daily dressing change, or silver-impregnated dressing changed every 3–5 d and excision and grafting
Full thickness	Topical antibiotics with twice-daily dressing change and excision and grafting

* Recommend restriction to individuals experienced with its use.

The importance of wound care—both at the time of initial debridement and at each dressing change thereafter—cannot be overemphasized. Wound care should be directed at thoroughly removing devitalized tissue, debris, and previously placed antimicrobials. A broad-spectrum surgical detergent such as chlorhexidine gluconate should be used for cleansing wounds during dressing changes. Adequate analgesia (e.g., frequent small doses of intravenous narcotics or ketamine), along with preemptive anxiolysis (e.g., preprocedure oral benzodiazepine), is necessary to permit adequate wound care. The most commonly used topical antimicrobials for the prevention and treatment of burn wound infection are mafenide acetate, silver sulfadiazine, silver nitrate solution, and silver-impregnated dressings.[52–54,58] Mafenide acetate and silver sulfadiazine are the topical agents typically available in the deployed environment. A brief review of each of these agents follows.

Mafenide Acetate

Mafenide acetate (Sulfamylon) was first introduced to burn care in 1964.[52] A retrospective study comparing USAISR Burn Center patients treated in the premafenide era (1962–1963) with those treated after the introduction of mafenide found a decrease in overall burn mortality from 38% to 20% and a reduction in the rate of invasive burn wound infection from 22% of admissions to 2%.[52]

Mafenide acetate is available as an 11% water-soluble cream composed of α-amino-p-toluenesulfonamide monoacetate. Despite the name, it is functionally a nonsulfonamide antibiotic. It rapidly penetrates full-thickness eschar and exerts a broad antibacterial effect.[59] In vitro and animal studies have demonstrated mafenide acetate to have efficacy against *Staphylococcus* and *Pseudomonas* species.[60,61] Although resistant strains of *Providencia* and *Enterobacter* developed at the USAISR in the late 1960s, none of the nearly 8,500 strains of *P. aeruginosa* isolated from USAISR burn patients during the period from 1967 to 1992 were resistant to clinically relevant concentrations of the drug.[62] There are some drawbacks to the use of mafenide acetate. It has no efficacy against filamentous fungi and induces pain on application, a consequence of its otherwise desirable ability to penetrate eschar and reach viable tissue. The drug and its primary metabolite (p-carboxybenzenesulfonamide) are inhibitors of carbonic anhydrase, and metabolic acidosis has been reported in patients with extensive burns treated twice daily.[63] Patients with inhalation injury are at greater risk for metabolic acidosis if their pulmonary dysfunction limits respiratory compensation.[63] This may pose a problem given that concentrations of the drug in eschar drop below therapeutic levels approximately 10 hours after application, necessitating twice-daily dosing unless a second agent is also used.[59] One common practice at the USAISR Burn Center is to apply mafenide acetate in the morning and silver sulfadiazine 12 hours later to realize the benefits of both drugs while limiting their toxicities.[62]

Mafenide acetate is also available in powder form for reconstitution as a 5% aqueous solution. This solution is used to moisten gauze dressings and is indicated for topical treatment of wounds after skin grafting. In addition, we often use this solution, along with twice-daily gauze dressing changes, for the topical treatment of deep partial-thickness burns of limited extent. However, this formulation has been shown to be less effective than mafenide acetate cream in preventing death in a murine model of *Pseudomonas* burn wound infection.[64]

Silver Sulfadiazine

Silver sulfadiazine (Silvadene, Thermazine, Flamazine, SSD, Burnazine) is available as a 1% water-soluble cream. It was developed in 1968 by complexing silver nitrate and sulfadiazine.[61,65] Previously, sulfadiazine alone had been used as a topical agent, but the development of resistance became an issue. Complexing sulfadiazine with silver nitrate has largely overcome the resistance problem, and the agents appear to act synergistically. In essence, the complex acts as a slow-release formulation of silver cation.[66,67] Much like mafenide acetate, silver sulfadiazine exhibits activity against gram-negative and gram-positive organisms; however, unlike mafenide, it has poor eschar penetration.[61,66,67] The advantages of silver sulfadiazine are that it is relatively painless on application and that it has some activity against *Candida* species (but not against filamentous fungi). Rarely, a decrease in the neutrophil count has been observed with initiation of therapy, attributed to depression of granulocyte macrophage progenitor cells in the marrow.[65] This effect typically resolves even when the agent is continued and rarely necessitates discontinuation of therapy.[65]

Silver Nitrate Solution

Silver nitrate ($AgNO_3$) solution was first introduced in 1964 as topical prophylaxis against burn wound infection. It had been previously used as a 10% solution that was found toxic to tissue.[67] It is now used as a 0.5% aqueous solution, a concentration which is not toxic to regenerating epithelium.[58,67] Burn wounds are dressed with multiple thick layers of coarse mesh gauze to which the silver nitrate solution is frequently reapplied to keep the gauze continuously moist.[62] Much like silver sulfadiazine, it exhibits activity against gram-positive bacteria, gram-negative bacteria, and *Candida* spp. The major drawbacks to silver nitrate solution are that it has poor penetration of eschar, requires the use of occlusive dressings, and turns black on contact with tissues.[67] Dressings must be changed twice daily to prevent buildup of exudate or of tissue-toxic levels of the silver nitrate. The need for continuously moist dressings means that patients with large wounds are at risk of hypothermia, particularly during transport or in general hospital rooms. Another drawback to this drug is the depletion of cations caused by leeching across the open wound into the hypotonic solution. This phenomenon may result in hyponatremia, hypocalcemia, hypokalemia, and hypomagnesemia; therefore, close monitoring of electrolytes is necessary.[58]

Silver-Impregnated Dressings

A variety of dressings impregnated with elemental silver have been approved by the US Food and Drug Administration (FDA) as topical therapy for burns. Several varieties of these dressings are now available, but heir equivalency in silver delivery and antimicrobial efficacy is difficult to assess.

Some examples of available silver dressings include Silverlon (Argentum LLC, Willowbrook, IL), SilverSeal (Noble Biomaterials, Scarnton, PA), and Acticoat (Smith and Nephew, Hull, United Kingdom). Silverlon is a knitted fabric composed of pure nylon-based fibers, covered uniformly and circumferentially with a thin coat of metallic silver. Alone and in combination with weak direct current, silver nylon has been shown to be effective in a lethal *Pseudomonas* murine model.[68] Acticoat is a rayon or polyester core encased in a dense polyethylene mesh coated with nanocrystalline silver. Tredget et al.[69] have reported Acticoat to be more effective than silver nitrate solution with respect to preventing heavy burn wound colonization (10^5 organisms per gram of tissue). Silverlon, SilverSeal, and Acticoat are approved for use in superficial and partial-thickness burns and can be left in place for several days thereby lessening the burden related to dressing changes. Clinicians should consider use of these agents for the treatment of wounds sufficiently small that outpatient or ward care are reasonable options.[70] The method of application for each of the topical agents is summarized in Table 3. T3

Excision and Grafting

Early excision of burned tissue and coverage with skin grafts or skin substitutes has been associated with a decrease in mortality among patients without concomitant inhalational injury.[16,17,20] The beneficial effect of this practice on mortality is likely multifactorial, with a decreased incidence of wound infection[18] and with the removal of devitalized tissue (which otherwise would prolong the inflammatory process) both likely playing a role. The definition of "early" excision has not been definitively established. Studies have variably defined early excision as that performed either at admission or up to 5 days after injury.[16,17,20] Early excision and grafting for deep partial-thickness and full thickness burns is recommended as soon as it is practical to do so. The accurate assessment of burn depth is challenging, and it is often difficult to predict the ultimate fate of a burn within hours to days of injury. In fact, some burns may progress from partial to full thickness during a period of days; thus, careful daily examination is critical.[74]

If excision is performed, the entire burn wound may be excised in a single procedure or in serial procedures performed during the course of several days.[55] Definitive coverage requires the application and successful integration of autograft. If sufficient autograft is not available, options for temporary wound coverage after excision include biological and synthetic coverings. Temporary biological dressings consist of allografts and xenografts. Allografts may be used to protect an excised wound or as an overlay to protect an excised wound after application of widely meshed (e.g., 3:1, 4:1) autograft. Fresh allograft may be available in the United States, but more often is frozen. A shelf-stable allograft product, GammaGraft, has been used in the combat zone during Operation Iraqi Freedom.[10] Xenografts (such as pig skin) are typically used as temporary coverage of wounds expected to heal.[74] Temporary synthetic skin substitutes are available. Biobrane is an example of a synthetic covering that is appropriate for clean partial-thickness burns. This, and similar products, act as a wound barrier and prevent evaporative losses but have no intrinsic antimicrobial properties.[55] Integra, a bilaminar product (inner dermal analog of chrondoitin-6-sulfate and collagen; outer temporary epidermal analog of silicone) should only be used by surgeons experienced in their use and under optimal conditions such as those available in a burn center.

As previously noted, surgical excision is normally not performed in the combat zone because it is labor- and supply-intensive and because optimal outcomes require the multidisciplinary capabilities present only in a burn center. However, definitive surgical care for local nationals may be required in the combat zone. We recommend that it be performed by qualified individuals at role 3 facilities,[75] recognizing that this situation is far from ideal.

CONCLUSIONS

The occurrence of invasive burn wound infection has decreased with the widespread use of topical antimicrobials, early excision and grafting, and the implementation of strict infection control measures in most centers. However, the uniquely austere environment encountered in the combat zone raises the issue of how best to prevent infection in injured military personnel. Wound care and the use of prophylactic topical antimicrobials should occur as soon as possible in the evacuation process. The use of systemic antimicrobials should be avoided during the evacuation process to minimize selective pressure for resistant organisms. Perioperative prophylaxis with systemic antimicrobials can be considered for excision and grafting procedures. The recommendations offered by this article will certainly evolve,

TABLE 3. Topical Antimicrobial Agents[41,58–63,65–67,71–73]

Agent	Application	Penetration	Side Effects
Mafenide acetate cream	Apply 1/16 inch layer twice daily*	Penetrates eschar	Painful on application, metabolic acidosis
Silver sulfadiazine cream	Apply 1/16 inch layer twice daily*	Poor eschar penetration	Transient leucopenia
Silver nitrate solution	Dress wounds with multiple layers of coarse gauze and apply solution to keep gauze continually moist	Poor eschar penetration	Electrolyte disorders
Acticoat, Silverlon, or Silverseal†	Moisten dressing with sterile water, cut to size, secure to wound with secondary dressing, change in 3–5 d	Poor eschar penetration	

* Consider alternating mafenide in the morning with silver sulfadiazine in the evening.
† Application information obtained from package insert.

along with our knowledge of the unique risks posed to the burn patient receiving initial care in the combat environment.

Research Gaps

Many gaps exist in our knowledge of the best methods of preventing and/or treating burn wound infections. As noted previously, a number of new dressing products which contain silver or (potentially) other antimicrobials have the potential to greatly facilitate wound care by permitting application earlier in the course of therapy, and by requiring far less frequent dressing changes with less pain, cost, and utilization of personnel. However, the ability of these agents to prevent infection in eschar-covered wounds appears to be limited but has not been studied adequately. The role of these topical agents in treating established wound infections is also not clear.

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Prevention of Infections Associated With Combat-Related Central Nervous System Injuries

AQ: 6

Michael A. Forgione, MD, FACP, Leon E. Moores, MD, FACS, Glenn W. Wortmann, MD, FACP, FIDSA, and the Prevention of Combat-Related Infections Guidelines Panel

Abstract: Combat-related injuries to the central nervous system (CNS) are of critical importance because of potential catastrophic outcomes. Although the overall infection rate of combat-related CNS injuries is between 5% and 10%, the development of an infectious complication is associated with a very high morbidity and mortality. This review focuses on the prevention of infections related to injuries to the brain or the spinal cord and provides evidence-based medicine recommendations from military and civilian data for the prevention of infection from combat-related CNS injuries. Prevention strategies emphasize the importance of expert evaluation and management by a neurosurgeon as expeditiously as possible. Areas of focus include elimination of cerebrospinal fluid leaks, wound coverage, postinjury antimicrobial therapy, irrigation, and debridement. Given that these recommendations are not supported by randomized control trials or adequate cohort studies in a military population, further efforts are needed to determine the best treatment strategies. This evidence-based medicine review was produced to support the "Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update" contained in this supplement of *Journal of Trauma*.

Key Words: Combat, Trauma, Central nervous system, Infection, Prevention.

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The prevention of infections associated with combat-related central nervous system (CNS) trauma is an important medical goal, as CNS infections are often catastrophic events. Battlefield injuries involving the head were reported in 6% of the 14,000 injuries evaluated and treated at the US 5th Army hospitals in 1944, with one-third of those classified as intracranial.[1] Penetrating spinal cord injury occurring on the battlefield, which perhaps most famously claimed the life of Admiral Horatio Nelson at the Battle of Trafalgar, was reported in nearly 12% of World War II battlefield injuries.[2,3] Combat-related CNS injuries are often associated with high-velocity weapons and/or blast injuries, creating substantial tissue destruction and frequently causing bone fragments and debris to be driven into the tissue.

Before World War I, penetrating head injuries were treated with expectant care. In a review of the historical treatment of head injuries, a mortality rate of 73.9% was reported in 898 cases of head wounds in the Crimean War and 71.7% in a series of 704 cases of penetrating head wounds from the American Civil War.[4] In 1918, Cushing found that >60% of deaths after dural penetration were because of sepsis. Without antimicrobial agents, he was able to reduce the mortality associated with CNS injuries from 54% to 29% simply by expediting surgical debridement.[5] The introduction of penicillin during World War II further helped decrease the mortality associated with CNS and spine trauma. Multiple reports from the 1940s described that the infection rates were between 21% and 31% with the use of parenteral sulfonamide therapy or locally applied sulfa powder; this rate improved to 5.7% to 13% with the use of penicillin.[1,6–9]

AQ: 1

Further improvements in medical and surgical care of combat-related CNS injuries saw mortality decrease to ~10% in the Korean and Vietnam wars and to 4.5% in 22 reported cases of head wounds during Operation Desert Storm.[10–13] As seen with combat-related penetrating head injuries, outcomes from penetrating spinal cord injury experienced a marked improvement with the introduction of antibiotics.[14] Although there has not been an official mortality figure, the current conflicts in Iraq and Afghanistan have resulted in the highest concentration of CNS injury among American warfighters since the Vietnam War. In a recent review of 513 patients with CNS injuries, >60% had additional multiple injuries and 46% had systemic infection which complicated their care.[15] Outcomes in this study showed that patients presenting with a Glascow Coma Scale score of 3 to 5 had a 24% mortality, compared with historical military averages of 50%.

METHODS

A MEDLINE search using PubMed from the US National Library of Medicine National Institutes of Health was performed using the key words "central nervous system," "military," "combat," "infection," "prevention," "spinal cord," "traumatic injury," "fixation," "irrigation," "debridement," "antibiotics," "ventricular catheters," "culture," "bac-

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AQ: 4

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AQ: 5

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terial," and "wound infection" with an emphasis on June 2007 through February 2011. We also cross-referenced published bibliographies for additional manuscripts.

EPIDEMIOLOGY AND MICROBIOLOGY OF WOUND COLONIZATION AND INFECTION

AQ: 2

Brain Injuries

There have been few studies reporting the bacteriologic culture of retained fragments or the identification of organisms associated with penetrating craniocerebral trauma. In 1942 at the Battle of El Alamein, Ascroft and Pulvertaft systematically obtained aerobic and anaerobic cultures from CNS traumatic injuries. The authors described 25 cases of penetrating craniocerebral injury, of which 6 cultures grew *Clostridium* spp., 22 grew *Staphylococcus aureus,* and 5 grew β-hemolytic streptococci in removed brain tissue; only 2 cases of sepsis resulted, both because of *S. aureus*.[16] In a study of brain wounds due to shell fragments during the Normandy campaign, Ecker performed bacteriologic studies on patients wounded 3 days to 86 days previously and who received sulfadiazine and penicillin. He found that 76% (32 of 42) of cultures grew organisms reported as *S. aureus* (7 cases), *Staphylococcus albus* (renamed *Staphylococcus epidermidis*) (17 cases), *Streptococcus viridans* (9 cases), nonhemolytic *Streptococcus* spp. (9 cases), gram-negative bacilli (6 cases), *Micrococcus tetragenus* (4 cases), and *Clostridium* spp. (2 cases).[17]

During the Vietnam War, Carey et al. took 45 craniocerebral missile cases and performed cultures of skin wounds, brain, and in-driven bone fragments within 4 hours of injury. They found skin wounds contaminated in 98% of cases, with 70% of the contaminating organisms being gram-positive cocci (predominantly *Staphylococcus* spp.) and 28% being various gram-negative rods. Only 11% of the brain wounds cultured showed bacterial contamination. In-driven bone fragments were positive in 20% to 45% of samples (depending on the number of bone fragments cultured) and all grew *Staphylococcus* spp. The authors concluded that skin bacteria were the most important source of contamination for cranial wounds, and that missile tracks within the brain were initially sterile.[18] Another Vietnam War paper reported that 35 of 62 (56%) patients operated on for retained intracranial bone fragments had positive microbial cultures of the fragment.[19] *S. epidermidis* was the most common organism isolated, with a variety of gram-negative and gram-positive bacteria also reported. The majority of patients in this report had undergone previous craniectomy and had been on antibiotics for a prolonged period of time (2 weeks).

Aarabi[20] reported 161 patients with missile head wounds injured in the Iran-Iraq war in 1987. In this study, all patients had received chloramphenicol with either ampicillin or penicillin G after field evacuation, and subsequently underwent culture of wound edges and brain tracks as well as all in-driven bone fragments. Wound cultures grew predominantly coagulase-negative *Staphylococcus* spp., whereas the brain tract cultures grew coagulase-negative *Staphylococcus* spp., *Acinetobacter* spp., and *S. aureus*. Cultures of bone fragments grew mostly coagulase-negative *Staphylococcus* spp. and *S. aureus*. Aarabi also reported six cases of meningitis (secondary to *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Herellea vaginicola*, *Enterobacter* spp., streptococci, and coagulase-negative *Staphylococcus* spp.) and two cases of brain abscess (coagulase-negative *Staphylococcus* spp. and *Escherichia coli*). Conclusions from this study showed no relationship between the contaminating bacteria and postdebridement infective organisms and that no patient with positive early wound, bone or brain culture, with or without bone or metal fragments retained, developed either meningitis or deep infection during follow-up.

Infection after combat-related penetrating brain injury is most commonly attributed to osteomyelitis of the skull, meningitis, or early or late abscess formation.[21] Studies from the Vietnam War era report a meningitis rate between 0.63% and 3.56% and a brain abscess rate of 2% to 3% in the patients.[11,19,22,23] Rish et al. in one of these studies describes 37 of 1,221 patients with culture-proven brain abscess after penetrating craniocerebral injuries. Of note, anaerobic culture data were not routinely obtained, with culture of gram-positive cocci (predominantly *S. aureus* and *S. epidermidis*) in 43% and gram-negative rods (a variety of organisms) in 56%.[22]

Several publications from the Croatian Homeland War outline infectious complications after penetrating brain injury. Hecimovic et al.[24] reported infectious complications occurring in 15 of 88 patients after missile brain injury (17%), with 14 of 15 patients developing an infection within the first 2 months after wounding. Of the infections noted, there were four cases of isolated bacterial meningitis, nine cases of brain abscess, one local cerebritis, and one subdural empyema with concomitant meningitis; *S. aureus* was the most commonly isolated organism. A 10% intracranial infection (meningitis, abscess) rate was reported by Vrankovic et al.[25] in their experience with 127 war-related missile brain injuries sustained in northeastern Croatia. In reporting complications of missile craniocerebral injuries during the Croatian Homeland War, Tudor et al.[26] found an 8.5% intracranial infection (meningitis, meningoencephalitis, or ventriculitis) rate in 176 patients. Finally, Splavski et al.[27] reported a 19% infection rate in 21 patients with skull base missile injuries describing three cases of brain abscess and one case of bacterial meningitis.

A series of 403 missile injuries to the brain during the Lebanese Conflict were reported by Taha et al.,[28] with an intracranial infection rate of 4.7%. More than 90% of infections occurred within 6 weeks of injury, and a mortality rate of 43% was observed. Gram-positive organisms were responsible for 36% of infections, gram-negative organisms accounted for 40%, mixed infections occurred in 7%, and 17% of cultures were negative. The authors concluded that the relatively high rate of gram-negative infections was attributed to the use of antibiotics before surgery. In 1990, Aarabi[29] reported the surgical outcome in 435 patients who sustained missile head wounds during the Iran-Iraq War. He found that 35 of 71 (49%) patients who died had an infection as a contributory factor (25 cases with meningitis and 10 with sepsis). Two differing reports outline the wartime neurosur-

gical experience in Lebanon. Levi et al.[30] reported a 4% intracranial infection rate in 116 patients, whereas Brandvold et al.[31] reported that 8% of their patients, injured in the same conflict, developed meningitis.

A recently published review from the current conflicts in Iraq and Afghanistan by Bell et al.[15] described 408 patients with closed or penetrating head trauma evaluated and managed at Walter Reed Army Medical Center or National Naval Medical Center in Bethesda, MD. In this study, 56% (228) of the patients had penetrating brain injury, with 71% of the penetrating injuries resulting from explosive blast injury and 24% due to gunshot wounds. Two hundred five patients required decompressive craniotomy because of the severity of their injuries. The authors also noted a 9.1% rate of meningitis in this population, with a rate of 26% in patients who had a concomitant cerebrospinal fluid (CSF) leakage from nose, ear, or wound. The microbiology of these infections included *Acinetobacter calcoaceticus-baumannii* complex (12), *S. epidemidis* (4), *Propionibacterium acnes* (4), *Enterobacter cloacae,* and *Enterococcus faecalis* (3), *Candida albicans* (2), *Staphylococcus capitis* (1), *Corynebacterium jeikeium*, (1) *E. coli* (1), and unspecified (9). The *Acinetobacter* infections were all carbapenem resistant and often resulted in multiloculated low pressure hydrocephalus. Most studies report that the majority of intracranial infections occur within 6 weeks of injury; however, delayed infection occurring years after the initial trauma has been reported.[32,33]

In summary, for penetrating brain injuries, study differences in culture techniques, prophylactic antibiotic use, and time of culture acquisition make definitive statements regarding the epidemiology of wound colonization and infection after injury difficult to conclude with certainty. Based on the available data, it seems that the most common organisms associated with wound colonization are of dermal origin (predominantly coagulase-negative *Staphylococcus* spp.). For intracranial infections, *S. aureus* or gram-negative facultative aerobic organisms are the predominant organisms. Intracranial infections occur more frequently when there is persistent CSF leakage after injury and, although most occur within several weeks of injury, can present in a delayed fashion.

Spinal Cord Injuries

Infectious complications occurring after spinal cord injury vary markedly in the literature, with meningitis being the most common. A report from the US military experience in Vietnam reported this complication in 6 of 19 (32%) patients sustaining a spinal cord injury secondary to a gunshot wound involving transcolonic injury.[34] Romanick et al. reported a similar rate of infection in a series of low-velocity missile wounds to the abdomen in a civilian institution. In this study, broad-spectrum antimicrobial therapy was used, and no patient without gastrointestinal tract perforation sustained infection.[35] Of the patients with colonic perforation, seven of eight patients developed infectious complications including one case of meningitis, three cases of abscesses, and three cases of osteomyelitis. Cultures from three of the patients grew *E. coli*, *Enterococcus* spp., and *Proteus mirabilis*, consistent with a colonic source of infection. Heary et al.[36] reported a 2% infection rate in penetrating spine trauma at a civilian institution. The infectious complications included meningitis (3), paravertebral abscess (2), vertebral osteomyelitis (1), and epidural abscess (1).

Other studies have reported contradictory results as to the risk of infection occurring after spinal injury. Waters and Adkins[37] reported no cases of meningitis or spine infection in 19 cases of spine injury associated with bowel injury. Kihtir et al.[38] reported no spinal or paraspinal infectious complications in five cases of spine injury with colonic injury. In a series of 42 patients with low-velocity gunshot wounds to the spine with an associated perforated viscous, Roffi et al.[39] stated that only 3 patients developed spinal or paraspinal infections. Two of 14 patients with colonic perforation developed psoas abscesses and 1 patient with a perforated stomach developed *E. coli* meningitis. A retrospective evaluation of 114 patients with low-velocity gunshot wounds to the spine demonstrated a significantly higher rate (14.8%) of both spine infections and wound infections when the gastrointestinal tract was involved.[40]

PREVENTION OF INFECTION

The prevention of infection after combat-related CNS injury will be discussed with focus on medical care capability from Role (also called level or echelon) 1 through Level III. Care at Role 1 includes immediate stabilization and evacuation. This care is started as close as possible to the time of injury. Role 2 care offers initial resuscitation and a short-term holding capability.[41] The US military can augment these facilities with a mobile surgical team able to provide lifesaving and sustaining surgical care. Role 3 care provides complete resuscitative and hospital care with a variety of medical and surgical capabilities.[42]

Initial care in the field for casualties with CNS injuries should focus on bandaging open wounds with sterile dressings to prevent further contamination. Dressings applied to open cranial and/or spinal injuries should provide protection while avoiding the placement of additional pressure on the exposed brain or spinal cord. Postinjury systemic antimicrobials should be administered as soon as possible after injury to prevent sepsis.

Several recent review articles have summarized data from civilian and military traumatic casualties resulting in penetrating brain injury and have recommended the use of postinjury antimicrobials for the prevention of infection.[21,43,44] The data supporting this recommendation are based on retrospective reviews and expert opinion, and do not support a standard treatment regimen or duration. For craniocerebral injuries, prevention of infection requires the use of antibiotics that treat *S. aureus* and gram-negative bacteria. In patients with penetrating spinal injury with involvement of the gastrointestinal tract, bowel flora should also be covered. Recommendations for postinjury antimicrobial therapy for penetrating brain and spine injury are cefazolin, 2 g given intravenously (IV) every 6 to 8 hours, with consideration of extending coverage with the addition of metronidazole 500 mg IV every 8 to 12 hours if gross contamination is present or the bowel is perforated. Alternative therapy includes ceftriaxone 2 g IV every 24 hours with consideration of extending

coverage with the addition of metronidazole 500 mg IV every 8 to 12 hours if gross contamination is present or the bowel is perforated. If the patient is allergic to penicillin, then vancomycin 1 g IV every 12 hours and ciprofloxacin 400 mg IV every 8 hours to 12 hours are recommended. Cefazolin and metronidazole were selected to maximize pharmacokinetics and pharmacodynamics for patients with multiple injuries, while simplifying the logistics for the combat zone. The use of high-dose cefazolin may also be weight-based as supported by recent pharmacokinetic studies.[45–47] The increased dosing interval is also supported by recent data.[48]

There are no controlled trials identifying the optimal duration of postinjury antimicrobial therapy following CNS trauma. A previous review has recommended 5 days of therapy for penetrating craniocerebral injury with retained organic material.[21] For penetrating injuries of the spine, one article suggested antimicrobial use for a minimum of 48 hours with extension to 7 days if the alimentary tract was violated.[36] A recent review of traumatic brain and spinal cord injury from the current conflicts in Iraq and Afghanistan revealed baseline rates of meningitis consistent with previous wars but noted a three times higher incidence of meningitis in patients with CSF leaks.[15] Based on the available literature, antimicrobial therapy should be continued for 5 days or until CSF leak control has occurred. Antimicrobials should be redosed following large volume resuscitation (estimated blood loss of 1,500–2,000 mL).[47,49–52] With ventriculostomy placement, it is common practice by many neurosurgeons to continue postinjury antimicrobials until final removal of these devices. A recent meta-analysis by Sonabend et al.[53] supported this practice, but the authors cautioned that the data supporting this finding are heterogeneous and suboptimal with further research needed to confirm the results of this analysis.

Craniotomy Versus Craniectomy

The choice of antibiotic regimen is not predicated upon the surgical decision to perform a craniotomy versus a craniectomy. If the mechanism of injury or intraoperative findings suggest that significant postoperative swelling may occur, it is common to leave the bone flap off, closing only the dura and skin—a craniectomy. Conversely, the absence of significant energy transfer, combined with intraoperative findings of minimal brain contusion or swelling, may allow the surgeon to directly replace the bone flap, thus performing a craniotomy. In either case, if the patient has a clean, uncontaminated wound, the standard antibiotic choice as described above would be appropriate. However, even if the lack of potential for swelling makes craniotomy appropriate, if air-filled sinuses were involved in the initial injury or gross contamination were present from a low energy penetrating injury, broadening the spectrum of antibiotic coverage in such a contaminated case would be recommended. Although it is reasonable to presume that a greater percentage of craniectomies may occur in the presence of contamination, it is the presence of the contamination and not the bone flap replacement (or absence thereof) which should dictate the choice of antibiotics.

DEBRIDEMENT AND IRRIGATION

Historically, extensive debridement of retained material had been recommended for penetrating brain injury; however, recent reviews have shown improved preservation of brain function with less aggressive surgical debridement.[54–59] Thus, current management is to remove only easily accessible foreign material and grossly devitalized tissue. Certain complications including CSF leaks, air sinus wounds, or wound dehiscence have all been identified as risk factors for infection and necessitate more aggressive surgical interventions.[22,60,61] There have been no studies evaluating the ideal irrigation fluid in CNS injuries. Based on current practices, room temperature normal saline, without additives, delivered under low pressure is commonly used.

For penetrating spinal injuries, retained bullets have not been shown to be a significant risk factor for infectious complications from low-velocity gunshot wounds unless the injury is associated with gross contamination or a tract exists from the peritoneal cavity to the spinal canal.[36] In patients with declining neurologic function, immediate removal of bone fragments or foreign bodies causing compression of neurologic structures is recommended to prevent further neurologic compromise, otherwise they can remain in place until evaluation by a neurosurgeon.[62]

TIMING OF WOUND CLOSURE

The injury site should be closed as quickly as possible, but with penetrating CNS trauma, there is often inadequate dura available for adequate closure. An autologous, vascularized, pericranial tissue graft or commercially available dural substitute can be used successfully in these instances. Cranialization of any violated sinuses and watertight dural and skin closure should follow adequate debridement.

In patients who have undergone aggressive cranial decompression after severe blunt or penetrating head injury, the removed bone flap should be discarded if the patient will ultimately be evacuated to a location where custom prosthetic implants are available.[63] This is the current US military practice. Other strategies used, where prosthetic implants are not available (e.g., for nonevacuated local nationals), include storage of the skull (bone) flap in the body (in subcutaneous abdominal tissue or the subgaleal space at the edge of the craniectomy) or in a freezer for later replacement. The best way to manage these bone flaps is not completely clear, but in a recent survey of 25 neurosurgical centers in Australia, cryopreservation was the most commonly performed technique for storage (88%).[64] This was typically performed using dry, sterile conditions with double or triple bagging (88%). Only 16% of hospitals irrigated skull flaps before storage (with either antibiotics in saline or Betadine). Storage temperatures ranged from -18°C to -83°C. Storage duration ranged from 6 months to indefinitely. The majority of centers (60%) screened the skull flaps by bacterial culture at the time of craniectomy.

The optimum timing for spinal fracture fixation is debated. Although studies have shown that fixation within 3 days can reduce the incidence of pneumonia, length of stay, number of ventilator days, and hospital charges, another

study demonstrated poorer outcomes in some groups with early spine stabilization.[65] The timing of fixation should be individualized, especially in those patients with other catastrophic injuries.[62]

UNRESOLVED ISSUES/FUTURE RESEARCH TOPICS

Although the use of antibiotics after penetrating brain and spine injuries has become standard-of-care, questions regarding the optimum choice of antibiotics and length of therapy are still unresolved. The use of prolonged antimicrobial prophylaxis with indwelling ventricular catheters needs to be evaluated in our patients with multiple injuries, as well as the applicability of antibiotic impregnated ventricular catheters. Improvements in the ability to rapidly diagnose wound infection versus colonization and further development of antimicrobial agents targeting MDR pathogens are needed at this time.

AQ: 3

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MINIREVIEW

Vaccine development in *Staphylococcus aureus*: taking the biofilm phenotype into consideration

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Abstract

Vaccine development against pathogenic bacteria is an imperative initiative as bacteria are gaining resistance to current antimicrobial therapies and few novel antibiotics are being developed. Candidate antigens for vaccine development can be identified by a multitude of high-throughput technologies that were accelerated by access to complete genomes. While considerable success has been achieved in vaccine development against bacterial pathogens, many species with multiple virulence factors and modes of infection have provided reasonable challenges in identifying protective antigens. In particular, vaccine candidates should be evaluated in the context of the complex disease properties, whether planktonic (e.g. sepsis and pneumonia) and/or biofilm associated (e.g. indwelling medical device infections). Because of the phenotypic differences between these modes of growth, those vaccine candidates chosen only for their efficacy in one disease state may fail against other infections. This review will summarize the history and types of bacterial vaccines and adjuvants as well as present an overview of modern antigen discovery and complications brought about by polymicrobial infections. Finally, we will also use one of the better studied microbial species that uses differential, multifactorial protein profiles to mediate an array of diseases, *Staphylococcus aureus*, to outline some of the more recently identified problematic issues in vaccine development in this biofilm-forming species.

A history of bacterial vaccines

The first bacterial vaccines developed used whole bacteria in either a live, attenuated vaccine (LAV) or a killed, whole-cell vaccine (KWCV). LAVs are generated either by repeat passage of the pathogen in a nonstandard host or in culture media or more recently by the targeted deletion of gene(s) that enable a pathogenic phenotype in humans. Louis Pasteur's work on the chicken cholera bacterium (*Pasteurella multocida*) and anthrax are the earliest examples of bacterial LAVs. Subsequent research on bacterial LAVs led to the development of the BCG vaccine for tuberculosis (Bastos *et al.*, 2009), the salmonella Ty21a vaccine for the prevention of typhoid (Wahdan *et al.*, 1980), and the CVD103-Hgr vaccine against cholera (Ketley *et al.*, 1993; Levine & Kaper, 1993). These vaccines continue to be used in developed and developing countries, because LAVs often confer a robust, long-lasting protection without the need to administer frequent booster shots.

Salmon and Smith subsequently laid the foundation for administering a heat-killed suspension of bacteria and paved the way for KWCVs. These vaccines were easy to produce, but had frequent adverse effects such as fever, anorexia, and swelling or induration induced by lipopolysaccharide. These drawbacks have led to almost complete clinical disuse of KWCVs in the United States. In response to these side effects, acellular, protein versions of traditional vaccines such as the acellular pertussis vaccines (Decker & Edwards, 2000) and the acellular anthrax vaccines (Friedlander & Little, 2009) followed. Rationales for immunizing with a limited number of antigens are reduced reactogenicity and avoidance of autoimmunity resulting from molecular

Table 1. General characteristics of classical bacterial vaccine types

Vaccine type	Pros	Cons
Killed, whole bacteria	Relatively simple to make Produces a protective immune response for many organisms	Highly reactogenic in many cases, this has rendered vaccines unusable or unpopular Risk of induction of autoimmunity via molecular mimicry Booster doses often needed
Live, attenuated bacteria	More robust and longer lasting immunity relative to killed, whole bacteria	Possibility of disease in immunocompromised patients Possibility of reacquisition of lost virulence resulting in disease Risk of secondary transmission
Toxoid	Excellent at generating toxin neutralizing antibodies Markedly less reactogenic compared with killed, whole bacteria	Multiple doses often needed Epitope must be highly conserved
Protein only	Markedly less reactogenic compared with killed, whole bacteria	Multiple doses often needed Epitope must be highly conserved
Polysaccharide only	Markedly less reactogenic compared with killed, whole bacteria	Multiple doses often needed Epitope must be highly conserved
Polysaccharide–protein conjugate	Improved antibody titers relative to polysaccharide only Decreased carriage for meningococcal and pneumococcal vaccines Can generate longer lasting immunity relative to polysaccharide vaccines Markedly less reactogenic compared with killed, whole bacteria	Meningococcal conjugate vaccine not currently recommended for children under age 11

mimicry by bacterial antigens (Zorzeto *et al.*, 2009). A limitation is that immunity elicited by a single antigen wanes more quickly than that generated by a LAV.

Alternatively, the tetanus and diphtheria toxoid vaccines developed in the 1920s are currently being used with minor alterations to their manufacture (Plotkin *et al.*, 2008). The toxoid vaccine lacks the toxin's pathogenic qualities and is used for vaccination to generate neutralizing antibodies against the toxin. Because single toxins are responsible for the bulk of *Clostridium tetani* and *Corynebacterium diphtheriae* pathogenesis, a robust immunoglobulin G (IgG) neutralizing antibody response that targets and blocks the toxin interrupts the disease process.

A better understanding of the critical role of polysaccharide capsules in the pathogenesis of *Streptococcus pneumoniae* and *Haemophilus influenzae* led to the development of polysaccharide vaccines (PSVs) against these pathogens (Riley *et al.*, 1977; Robbins *et al.*, 1983; Mufson *et al.*, 1985) as well as a PSV against *Neisseria meningitidis* serotypes A, C, W-135, and Y (Artenstein *et al.*, 1970; Armand *et al.*, 1982; Ambrosch *et al.*, 1983). Because of suboptimal immunogenicity elicited by polysaccharide, PSVs are being eliminated and replaced by polysaccharide–protein conjugate vaccines. Conjugate vaccines elicit a robust IgG response imparted by the protein carrier, which converts the polysaccharide from a T-cell-independent immunogen into a T-cell-dependent immunogen (Perez-Melgosa *et al.*, 2001).

Innovations to vaccine design over the years have resulted in a number of successful bacterial vaccines that supplant earlier, less effective vaccines. Currently, several competing cholera (Lopez *et al.*, 2008) and typhoid vaccines (Fraser *et al.*, 2007) are available. A closer examination of these vaccines defines the pros and cons of certain vaccine strategies (Table 1).

Although vaccinology has made significant progress (Table 2), many challenges remain to date. When dealing with bacterial pathogens that can cause multiple forms of diseases through a large number of virulence factors, often traded between individual strains and species by horizontal gene transfer, protection via a single component vaccine is likely to be elusive. *Staphylococcus aureus* is an example of such a pathogen. This microbial species has dozens of known toxins, multiple immunoavoidance, and adherence factors, most of which demonstrate transient, timed, and disease-specific expression (DeLeo *et al.*, 2009). Therefore, a successful vaccine will likely be required to provide protective antibody titers against multiple antigens (Zecconi *et al.*, 2005).

Types and modes of delivery of vaccines

Recombinant subunit protein technology has become the main strategy in the development of vaccines against infectious diseases. Subunit vaccines offer several advantages over previous vaccine strategies. Recombinant subunit vaccines are safe or less reactogenic with a defined composition, which is due to its genetic-based approach and antigen expression in nonpathogenic bacterial strains. Other advantages include multiple modes of delivery and further engineering of the subunit (Liljeqvist & Stahl, 1999; Hansson

Table 2. Common bacterial vaccines

Pathogen (disease)	Vaccine type	Composition	Current status
Bacillus anthracis (anthrax)	Live, attenuated	Sterne live-attenuated strains	Not available in the United States for humans, only for veterinary use
	Acellular	Cell-free culture supernatant adsorbed to aluminum hydroxide; believed to contain mostly the protective antigen of the anthrax toxins	Not available to the public in the United States
Bordetella pertussis (pertussis)	Killed, whole cell	Killed pathogenic bacteria	Completely replaced by acellular vaccine in the United States and many developed countries
	Acellular	Inactivated pertussis toxin plus one or more of the following proteins: hemaglutinin, pertactin, or fimbriae types 2 and 3	Approved for clinical use in the United States
Borrelia burgdorferi (Lyme disease)	Killed, whole cell	Inactivated whole-cell vaccine with proprietary polymer adjuvant or bivalent whole-cell killed	Veterinary vaccines for dogs
	Lipoprotein	Lyme OspA recombinant lipoprotein	Withdrawn from clinical use in 2002
Clostridium tetani (tetanus)	Toxoid	Formaldehyde detoxified tetanus toxin	Currently licensed in the United States in several combinations
Corynebacterium diphtheriae (diphtheria)	Toxoid	Diphtheria toxoid adsorbed to aluminum salt	Currently licensed in the United States in several combinations
Coxiella burnetii (Q fever)	Killed, whole cell	Killed *C. burnetii*	Not commercially available in the United States
Haemophilus influenzae type B (pneumonia and meningitis)	Polysaccharide	Polyribosylribitol phosphate (PRP)	Not effective in children younger than 18 months (the population that experiences the most severe disease), not currently used in the United States
	Polysaccharide–protein conjugate	PRP or HbOC linked to either diphtheria toxoid or the outer membrane protein complex of *N. meningitidis*	Four currently licensed conjugate vaccines in the United States
Mycobacterium tuberculosis (tuberculosis)	Live, attenuated	Bacille Calmette-Geurin (BCG)	Widespread global use; rarely administered in the United States
Neisseria meningitidis (meningitis)	Polysaccharide–protein conjugate	Quadrivalent vs. A, C, Y, and W-135 strains	Currently licensed in the United States
Rickettsia rickettsii (typhus)	Killed, whole cell	Inactivated chick embryo cultured *R. rickettsii*	No currently licensed vaccine in the United States
Salmonella typhi (Typhoid)	Killed, whole cell	Heat- and phenol-inactivated *S. typhi*	No longer available in the United States
	Killed, whole cell	Acetone inactivated parenteral vaccine	Only available to the United States Armed Forces
	Live, attenuated	Ty21a galactose nonfermenting *S. typhi*	Available in the United States
	Polysaccharide	Vi capsular antigen	Available in the United States
	Polysaccharide–protein conjugate (Vi-rEPA)	Vi capsular antigen conjugated to *Pseudomonas aeruginosa* recombinant exotoxin A	In development
Streptococcus pneumoniae (pneumonia and meningitis)	Killed, whole cell	Monovalent killed	Abandoned, not available
	Polysaccharide	6-, 14-, and 23-valent polysaccharide vaccines	No longer used in the United States because it couldn't be used for children < 2 years old and superior protection was afforded by conjugate vaccines
	Polysaccharide–protein conjugate	7-valent polysaccharide conjugated to diphtheria CRM_{197} carrier protein	Currently licensed for prevention of infant and child meningitis
	Polysaccharide	23-valent polysaccharide	Licensed for the prevention of pneumonia in patients of 65 years of age or older or immunosuppressed patients over the age of two
Vibrio cholerae (Cholera)	Killed, whole cell	Killed pathogenic bacteria	Licensed, but not widely used

Table 2. Continued.

Pathogen (disease)	Vaccine type	Composition	Current status
	Killed, whole cell plus recombinant protein (WC-rBS)	Two heat-killed strains of *V. cholerae* plus recombinant cholera toxin B	Only approved for experimental use in the United States
	Live, attenuated (CVD103-Hgr)	Pathogenic bacteria with the cholera toxin B subunit deleted	Only approved for experimental use in the United States
Yersinia pestis (Plague)	Killed, whole cell (Haffkine vaccine)	Heat-inactivated whole organism	Generated severe AE's, never widely adopted
	Killed, whole cell	Formalin-inactivated *Y. pestis*	Formerly licensed for sale and used in military personnel during Vietnam War; no longer available due to marked AE's to initial and booster doses

AE, adverse event; HbOC, *Haemophilus* b oligosaccharide conjugate (derivative of PRP); PRP, polyribosylribitol phosphate.

et al., 2000). The main drawbacks of subunit vaccines are the requirement of an adjuvant and multiple doses as well as low immunogenicity and a short half-life, which can be improved by conjugating the protein subunit to another protein or molecule (Hudecz, 2001; Tugyi *et al*., 2008). Conjugation of an antibody, adhesion factor, or other molecule (such as cholera toxin B subunit) to the peptide can target it to immunologically relevant sites or cells to improve response. Recombinant subunit vaccine efficacy is also reliant on the route of administration.

Current delivery methods include parenteral (e.g. transcutaneous and intramuscular) and mucosal (e.g. intranasal and oral) vaccines. The skin serves as a functional barrier by preventing harmful molecules and organisms from invading the host. Langerhans cells, a class of antigen-presenting cells, present antigens in the epidermal layer and the accessibility of the skin makes parenteral vaccination a favorable delivery method. The parenteral route of vaccine delivery is an effective inducer of systemic immunity represented by significant serum IgG titers and cytokine expression in lymph nodes. Nevertheless, this mode of vaccine delivery is deficient in its ability to initiate a mucosal immune response.

The mucosal surface is resident to the majority of lymphocytes found in the human body and is also the main entry point for infectious agents. This makes targeting vaccines to the mucosal sites crucial for immunity. The main advantage of mucosal vaccination over parenteral is the induction of IgA secretion at mucosal sites in combination with systemic IgG titers. Secreted IgA prevents the colonization and invasion of pathogens and neutralizes toxins at the mucosa (Slutter *et al*., 2008). Mucosal vaccination leads to antigen-specific B cell memory, with the caveat that a proper immunostimulating compound is used (Vajdy, 2006). Antigen delivered without an adjuvant leads to mucosal tolerance, resulting in clonal deletion or induction of anergy of antigen-specific lymphocytes (Ogra *et al*., 2001). In addition to mucosal tolerance, inefficient uptake of antigen and delivery to antigen-presenting cells is another disadvantage of mucosal vaccination (Slutter *et al*., 2008). Mucosal vaccination has the potential to alleviate the innumerable diseases caused by pathogenic bacteria, viruses, and parasites by providing complete protection through IgA-mediated mucosal and IgG-mediated systemic immunity. Overcoming the hurdles of mucosal tolerance and inefficient antigen delivery may augment the vaccines currently in clinical trials.

Adjuvants

Adjuvants work by stimulating the innate immune response, which is a required step in activating adaptive immunity. Cytokines and chemokines expressed upon stimulation of the innate immune response attract leukocytes to the local environment and cause maturation of antigen-presenting cells such as dendritic cells (DCs). The resident DCs are effective messengers between the innate and the adaptive response due to their enhanced antigen-presenting capabilities and ability to become polarized. Adjuvants promote cytokine expression within a microenvironment that polarizes DCs to mediate the expression of Th1 or Th2 cytokines and costimulatory molecules. In the draining lymph nodes, polarized DCs present the antigen to naïve T-cells. The development of Th0 to Th1, Th2, or other T-helper cells during antigen presentation is dependent on the expression of polarizing cytokines and costimulatory receptors produced by DCs. T-cells activated during this process potentiate the subsequent adaptive immune response.

Selecting the appropriate adjuvants for vaccine development is crucial, because they play a critical role in the development and polarization of the adaptive immune response. Adjuvants have been found to favor either a Th1 or a Th2 response, suggesting the production of Th1- and Th2-polarizing cytokines at the site of administration. To

understand the immune response initiated by an adjuvant, whether it be Th1 or Th2, becomes essential in the selection of an adjuvant for vaccine design. Few adjuvants exist in the clinical realm; however, many are being tested experimentally. Table 3 details supplemental information on the current and experimental adjuvants.

Adjuvants are potent inducers of innate immunity. They are often needed for an effective and protective adaptive immune response against pathogens. The Th response stimulated by vaccination is dependent on the cytokine milieu produced locally by an adjuvant, and the resultant polarization of antigen-presenting cells. Also, planktonic vs. biofilm-mediated diseases initiated by the same pathogen complicate vaccine development as each phenotype may require different Th responses to provide postvaccination protection. Research on the immunostimulating properties of molecules will elucidate future adjuvants and provide even greater options for vaccine development.

Novel strategies for antigen selection: highlighting *S. aureus* advances

Vaccine design changed dramatically with advancements in genome sequencing technologies that enable rapid completion of genomes. Since the publication of the *H. influenzae* genome in 1995, the NCBI genome project reports that 1026 complete microbial genomes have been published including ones for 15 *S. aureus* strains (Fleischmann *et al.*, 1995) (http://www.ncbi.nlm.nih.gov/genomeprj). Access to complete genomes and bioinformatic technologies to manage and analyze the data has advanced high-throughput molecular techniques for genomic, transcriptomic, and proteomic analyses of microbial growth and pathogenesis (Kaushik & Sehgal, 2008; Zagursky & Anderson, 2008). Genome-based technologies provide rapid identification of vaccine candidates compared with the conventional vaccine approaches, which identify and analyze individual virulence factors from pathogens grown *in vitro* (Rappuoli, 2000). Vaccines developed via genome-based technologies will still slowly transition into clinical phases after rapid identification, because these vaccines require the same rigorous evaluations using *in vitro* assays and animal models to validate functional activity as conventionally derived vaccines. As this review focuses on vaccine development against *S. aureus* to highlight *in vivo* phenotypes (e.g. biofilm formation and polymicrobial infection) that should be considered during antigen identification, we choose to present genome-based strategies and other technologies that identified putative *S. aureus* virulence factors and/or vaccine candidates. Vaccines comprised of antigenic candidates identified by these strategies may provide protection against *S. aureus* infection, but the overall lack of an effective *S. aureus* vaccine to date indicates that critical phenotypes and factors are not adequately addressed in current vaccines. For the strategies outlined below, both these and future studies examining alternate parameters will

Table 3. Adjuvant-dependent effector T cell differentiation

Adjuvants	Clinical status	Immune response	Experimental observations to designate immune response		References
Alum	Only one approved for US vaccines	TH2	TH1	No IgG2a titer No IFN-γ	Uddowla *et al*. (2007) Brewer (2006)
			TH2	High IgG1 titer IL-4 and IL-5 produced	Uddowla *et al*. (2007) Brewer (2006)
MF59	Fluad influenza vaccine*	TH2	TH1	Low IgG2a titer	Valensi *et al*. (1994), Wack *et al*. (2008)
			TH2	High IgG1 IL-5, IL-4, and THF-α produced	Valensi *et al*. (1994), Wack *et al*. (2008)
MF59 with CpG	No clinical application†	TH1	TH1	High IgG2a titer IFN-γ produced	Wack *et al*. (2008)
			TH2	Low IgG1 titer IL-5 suppressed	Wack *et al*. (2008)
AS04	Cervarix* (HPV)–Fendrix* (Hepatitis B)	TH1	TH1	High IgG2a IL-2 and IFN-γ produced	Korsholm *et al*. (2010), Didierlaurent *et al*. (2009)
			TH2	Low IgG1 IL-6 and THF-α produced	Korsholm *et al*. (2010), Didierlaurent *et al*. (2009)
c-di-GMP	No clinical application†	TH1/TH2	TH1	High IgG2a and IgG2b IFN-γ, THF-α, IL-12, MCP-1, and RANTES produced	Karaolis *et al*. (2007), Hu *et al*. (2009)
			TH2	High IgG1 and IgG3	Hu *et al*. (2009)

*European-approved vaccine application only.
†Not approved for human vaccine applications.

be invaluable resources to refine the search for vaccine candidates.

Genomics/transcriptomics

Identification of vaccine candidates through the systematic search of the genome and identification of putative antigens, mainly surface-associated proteins, using bioinformatics is referred to as 'reverse vaccinology' (Rappuoli, 2000). The progression of this field and its significance to vaccine development against serogroup B *N. meningitidis* and group B *Streptococcus* are detailed in reviews by Serruto & Rappuoli (2006), Serruto *et al.* (2009). This method has a number of advantages compared with previously used methods in that there is no need to grow the pathogen *in vitro* and antigen selection can proceed independent of the abundance of *in vivo* expression and immunogenicity. As a result, many unique antigens can be tested that would have been passed over in conventional studies.

Vaccine candidates identified from a single genome in reverse vaccinology must provide *in vivo* protection against multiple clinical strains in correlative animal models to support transition into clinical studies. An approach, known as comparative genomic hybridization (CGH), uses a DNA microarray of a sequenced 'reference' strain to screen for the presence or absence of genes within nonsequenced 'test' strains and limits the candidates to antigens conserved in multiple strains. However, the modern ability of advanced sequencing methods such as pyrosequencing has enabled whole-genome sequencing for multiple genomes from various strains of a microbial species to become commonplace. Access to complete genomes of multiple strains for some bacteria makes sequence comparisons among multiple genomes a favorable alternative to CGH because the comparison accounts for all genes within each strain. Earlier CGH studies and more recent deep strain sequencing have led to a description of the 'pangenome' in three parts: a 'core' genome comprised of genes conserved in all genes, a distributed genome composed of genes not conserved in one or more strains, and a subgroup comprised of novel genes encoded by a single strain (Tettelin *et al.*, 2002, 2005; Shen *et al.*, 2005; Ehrlich *et al.*, 2008). A protective quadrivalent vaccine for *S. aureus* was assembled from surface proteins, IsdA, IsdB, SdrD, and SdrE, after searching eight genomes and evaluating the protective efficacy of multiple candidate antigens in mice (Stranger-Jones *et al.*, 2006).

The increased access to complete genomes of bacteria has led to the ability to develop unique cDNA microarrays for transcriptomic profiling. Evaluation of the bacterial transcriptome under *in vitro* conditions, mimicking environmental stimuli encountered during host infection, detects upregulated genes that may represent virulence factors and vaccine candidates. Transcriptomic analysis is generally restricted to *in vitro* studies, because bacterial RNA is difficult to extract differentially from the infected host tissue.

Gene expression technologies: positive selection

Other technologies make use of the *in vivo* transcriptional profiles to gather information on the genes involved in virulence, but circumvent the restrictions of RNA extraction and microarray analysis. Three techniques that analyze *in vivo* gene expression and predict promising vaccine candidates are *in vivo* expression technology (IVET), differential fluorescence induction (DFI), and *in vivo* induced antigen technology (IVIAT) (Mahan *et al.*, 1993; Valdivia & Falkow, 1996; Handfield *et al.*, 2000).

The first report of IVET applied to a Gram-positive species was a study of *S. aureus* by Lowe *et al.* (1998), using a variation known as recombination-based IVET (RIVET). In the RIVET system, random genomic fragments are fused to a promoterless resolvase gene, such as *tnpR*, to construct a genomic library, and a gene cassette comprised of an antibiotic resistance gene flanked by resolvase recognition sequences is incorporated into the bacterial genome. Excision of the antibiotic marker from the bacterial genome, or 'resolution', is dependent on the expression of the *ivi* gene-resolvase fusion, and confers antibiotic sensitivity to the bacterium (Angelichio & Camilli, 2002). Lowe *et al.* (1998) assessed 11 mutants for *ivi* genes that were identified from *S. aureus* genomic libraries screened in a murine renal abscess model and defined seven mutants with attenuated virulence compared with wild-type *S. aureus*. DFI is another promoter-trap approach where promoter induction controls the expression of green fluorescent protein, and microorganisms with gene expression can be isolated by fluorescence-activated cell sorting (Valdivia & Falkow, 1996). Finally, the IVIAT system screens *in vitro* expression libraries of a pathogen with convalescent sera following depletion of antibodies specific to that pathogen grown under *in vitro* conditions.

Gene expression technologies: negative selection

Signature-tagged mutagenesis (STM) identifies the genes required for *in vivo* growth and survival by screening heterogeneous pools of mutants. Each of the mutants has a transposon with a unique oligonucleotide tag randomly incorporated into their genome. After inoculating pools of mutants into a relevant *in vivo* infection model, those mutants that fail to colonize the model can be identified by their unique transposon tag (Hensel *et al.*, 1995). STM screens of *S. aureus* virulence in murine models of bacteremia, abscess, and wound and rabbit endocarditis have been

completed, and report that $< 10\%$ of the mutants were attenuated in all three murine models (Mei *et al.*, 1997; Coulter *et al.*, 1998).

Proteomics

Proteomic profiling examines and identifies the spectrum of proteins expressed in bacteria under varying growth conditions using two-dimensional gel electrophoresis (2DGE) and MS. Detection of membrane and cell wall proteins is a limitation of proteomic profiling due to low abundance and solubility constraints that are caused by protein hydrophobicity, transmembrane domains, and an alkaline isoelectric point (Fountoulakis & Takacs, 2001). Because vaccine strategies focus on surface-associated proteins, proteomic analyses yield limited vaccine candidates unless extraction protocols that solubilize membrane proteins or isoelectric focusing performed in the alkaline pH range are used. Reference maps of *S. aureus* Phillips and VISA surface proteomes following lysostaphin extraction have been published, and among these, membrane- and cell wall-associated proteins are promising candidate antigens that can be tested for immunogenicity and/or protective activity (Nandakumar *et al.*, 2005; Gatlin *et al.*, 2006). Another strategy, considered a 'new chapter in reverse vaccinology,' developed concurrently with the cited work of Nandakuman and colleagues, and Gatlin and colleagues examined surface proteins 'shaved' from group A *Streptococcus* using trypsin disgestion (Musser, 2006; Rodriguez-Ortega *et al.*, 2006). Cell surface shaving proteomics has recently established 42 *S. aureus* COL surface proteins that may have potential for vaccine development (Solis *et al.*, 2010).

Serological probing of proteomic samples, known as immunoproteomics, followed by peptide identification using matrix-assisted laser desorption ionization time-of-flight MS is a direct method for defining antigenic proteins. An initial 2DGE immunoproteomic study of *S. aureus* COL identified 15 known and novel proteins that were immunoreactive with patient sera (Vytvytska *et al.*, 2002). Using subtractive proteome analysis, Glowalla and colleagues selected proteins that were immunoreactive with an intravenous immunoglobulin (IVG) preparation and nonreactive with IVG depleted of *S. aureus*-specific opsonizing antibodies and identified three anchorless cell wall proteins that provided partial protection in a mouse sepsis model (Glowalla *et al.*, 2009). These anchorless wall proteins lack a conserved signal peptide or an LPXTG motif, characteristic of most surface-associated proteins, and in some cases, may be consequently omitted from classical reverse vaccinology screens (e.g. vaccine development from genome analysis) (Chhatwal, 2002). Immunoproteomic studies have also evaluated two obstacles to the clinical control and prevention of *S. aureus*, biofilms that potentiate chronic infections and colonization or human carriage (Brady *et al.*, 2006; Holtfreter *et al.*, 2009). Indeed, most humans possess preexisting circulating antibodies against major *S. aureus* virulence factors that do not protect against a subsequent challenge by this pathogen. Incomplete protection may be attributed to the transient nature of virulence factor expression during the infection, which requires consideration during the process of vaccine development.

Antigenomics

Antigenomic screens probe *Escherichia coli* surface-expressed fusions that express randomly fragmented genomic libraries with human sera that are depleted of *E. coli*-specific antibodies. The screens identify a large repertoire of antigenic peptides including those encoded by alternate reading frames (Etz *et al.*, 2002). Indeed, antigenomic studies of *Staphylococcus* and *Streptococcus* found that 24% of antigens were hypothetical proteins or proteins of unknown function from nonannotated reading frames (e.g. alternative reading frame, complementary strand reading frame, nongene matching reading frame), which are categories eliminated from bioinformatics-based vaccine development (Meinke *et al.*, 2005). Antigenomic peptides can be evaluated for widespread *in vivo* expression, or reactivity, via screening with multiple serum samples and conserved expression among multiple bacterial strains (Etz *et al.*, 2002). High-throughput screening methods that circumvent the restrictive in-frame cloning step and peptide insolubility issues that limit peptide repertoire in the bacterial surface expression systems include phage display and ribosome display. However, antigenomic strategies may inadequately define antigenic peptides compared with *in vitro* expression systems, possibly due to protein toxicity and reduced membrane permeation obstructing surface expression and limiting antigen detection.

Taking into account the mode of growth: biofilm vs. planktonic

The early pioneering work and the continued modern era of biofilm disease discovery by a number of investigators have transformed the field of medical microbiology (Nickel *et al.*, 1985a, b, 1986a, b, 1989; Post *et al.*, 1996; Ehrlich *et al.*, 2002; Erdos *et al.*, 2003; Murphy *et al.*, 2005; Stoodley *et al.*, 2005; Hall-Stoodley *et al.*, 2006; Hiller *et al.*, 2007; Hogg *et al.*, 2007). Because of these studies, the biofilm mode of growth has been recognized as the major mode of infection, with an estimated 80% of all infections caused by biofilms (National Institutes of Health, 1998, 1999). Although extensive studies have been performed on biofilm infections, the resolution of these infection continues to be the surgical removal of the nidus of infection (Shirtliff & Mader, 2000). This surgical removal is necessary because these microbial

communities are 50–500 times more resistant to antimicrobial agents than their planktonic and free-floating counterparts (Nickel *et al.*, 1985b; Stewart & Costerton, 2001). Although the significance of biofilm infections has been recognized as an important mediator of chronic infection and the resulting morbidity and mortality, vaccine studies have often ignored biofilms in discovery and efficacy studies.

For example, recent vaccine development programs for *S. aureus* have tended to focus on testing the ability of target antigens to protect the host from *in vitro* or murine planktonic infection models (Fattom *et al.*, 1996, 2004; McKenney *et al.*, 1998, 1999, 2000; Stranger-Jones *et al.*, 2006; Bubeck Wardenburg & Schneewind, 2008; Lin *et al.*, 2009; Kim *et al.*, 2010). Infections with *S. aureus* may exist in a biofilm mode of growth either during nares carriage or skin infections. Once transmitted to the circulatory system through an epithelial breach, planktonic growth ensues, where upregulation of adherence factors occurs (Beenken *et al.*, 2004). At this point, the invading staphylococci are either removed by the host innate immune response or attach to host extracellular matrix proteins and develop a localized biofilm community. Once this community develops, the proteome of the microorganisms quickly transforms into a biofilm phenotype. Therefore, the planktonic mode of growth that occurs in sepsis may be a transient state. Also, although the host may be vaccinated against planktonic antigens, they may develop a significant memory response only after the secondary foci of biofilm infection has already occurred and the antigenic nature of this pathogen has also significantly changed, thereby detracting from vaccine efficacy.

In the context of biofilm infections, the first question that must be answered when selecting antigen targets is which component of the biofilm should be targeted. Broadly speaking, two alternatives exist: bacterial cells within the biofilm and the biofilm matrix itself. The biofilm matrix may be composed of polysaccharides, protein, or extracellular DNA, in proportions that vary between bacterial genera, species, and strains. As of 2009, the majority of antibiofilm vaccine efforts have been directed toward the biofilm matrix (Schaffer & Lee, 2008). Perhaps the best example of this is the staphylococcal polysaccharide intercellular adhesin (PIA), which is composed of poly-*N*-acetyl-β-1,6-glucosamine (PNAG). The enzymes that catalyze the production of these polysaccharides are encoded for by the genes of the *icaADBC* locus (Joyce *et al.*, 2003). PIA is produced by both *Staphylococcus epidermidis* (McKenney *et al.*, 1998) and *S. aureus* (Cramton *et al.*, 1999), and is known to be involved in the adherence of *S. epidermidis* to both host tissues (Costa *et al.*, 2009) and inert biomaterials (Olson *et al.*, 2006). PIA/PNAG plays an additional role in immune evasion in both the biofilm and the planktonic mode of growth. The *icaADBC* locus has been detected in clinical *S. epidermidis* isolates (Ziebuhr *et al.*, 1997), and its contribution to pathogenesis has been demonstrated in animal models of infection (Rupp *et al.*, 1999). Hence, upon a superficial review, PIA would seem to be an ideal candidate for a vaccine antigen.

In contrast to *S. epidermidis*, PIA production is less pronounced in most *S. aureus* strains and often observed *in vitro* only under particular conditions, such as anaerobiosis (Cramton *et al.*, 2001) or relatively high (1%) glucose concentrations (Ammendolia *et al.*, 1999). In one study, only 57% of strains that were *icaADBC* positive by PCR analysis (Arciola *et al.*, 2001a) produced a biofilm when cultured *in vitro* (Knobloch *et al.*, 2002), suggesting distinct strain differences in any correlation of PIA and biofilm formation. *In vivo*, analysis of clinical *S. aureus* isolates from prosthetic-joint infections, bacteremia (Fowler *et al.*, 2001), catheter-related infections (Arciola *et al.*, 2001a), or from randomly selected clinical isolates (Martin-Lopez *et al.*, 2002) indicates possession of the *ica* locus by the majority of isolates. However, a lack of PIA production was observed in many of these strains *in vitro*. The proportion of *ica*-positive strains among *S. aureus* clinical isolates is thought to vary according to the clinical origin of the isolate and even between infection sites that are both biofilm mediated. For example, the proportion of *icaADBC*-positive *S. aureus* strains was higher in orthopedic prosthesis-associated infection (92%) than in catheter-associated infections (63%) (Rohde *et al.*, 2001). Thus, the site and composition of indwelling biomaterials may act as selective factors for strains with different and alternate adhesion mechanisms. The situation is further complicated by the fact that possession by a staphylococcal strain of the *icaADBC* locus does not necessarily mean that PIA will be produced *in vivo*. Similarly, the production of PIA *in vitro* does not mean that it will be produced *in vivo* during an infection. In addition, *in vitro* PIA expression may differ between assays (Rohde *et al.*, 2001). Although there is some evidence that suggests a correlation between *icaADBC* possession and slime production *in vitro* (Arciola *et al.*, 2001b), more research is required to fully understand the importance of PIA in staphylococcal infection *in vivo*. There is also limited evidence that suggests that PIA expression can undergo phase variation (Ziebuhr *et al.*, 1997).

A vaccine based on PIA has undergone trials in animal models. McKenney *et al.* (1998) used PNAG to immunize mice. Five days after an intravenous challenge with two *S. aureus* strains (CP5 Reynolds and CP8 MN8), both of which are negative for PNAG production *in vitro*, immunized mice showed a significant reduction in CFU recovered from the kidneys as compared with the controls (McKenney *et al.*, 1999). Further work by the same group suggested that the deacetylated form of PNAG, dPNAG (15% acetylation), conjugated to the diphtheria toxoid is more effective as a

vaccine than the 90% acetylated form (Maira-Litran *et al.*, 2005). This is likely due to the retention of dPNAG on the bacterial cell surface, in contrast to the highly acetylated PNAG form, which is released into suspension (Cerca *et al.*, 2007). The deacetylase activity of the *icaB* gene product (Vuong *et al.*, 2004) mediates this effect. The use of PNAG as a vaccine has shown promise in subsequent studies in animal models of *S. aureus* mastitis (Perez *et al.*, 2009) and *S. aureus* skin abscess (Gening *et al.*, 2010). Given that PNAG is produced by a variety of other bacterial taxa, including *E. coli* (Wang *et al.*, 2004), *Actinobacillus actinomycetemcomitans*, *Actinobacillus pleuropneumoniae* (Kaplan *et al.*, 2004), *Bordetella* spp. (Parise *et al.*, 2007), and *Acinetobacter baumannii* (Choi *et al.*, 2009), PNAG has shown promise in subsequent vaccine studies in animal models of *E. coli* bacteremia (Cerca *et al.*, 2007) and peritonitis (Gening *et al.*, 2010).

The efficacy of a PNAG-based vaccine against *S. aureus* biofilm-type infection remains to be elucidated. However, given that possession of the *icaADBC* locus by clinically isolated *S. aureus* varies between infection sites (Rohde *et al.*, 2001), PNAG may not be the ideal vaccine antigen in a formulation intended to prevent biofilm-type infections. Besides PIA/PNAG, other biofilm factors have simply not been evaluated extensively and these may potentially be inappropriate targets in subsequent studies. Also, one may question whether it would be more efficacious to promote the host immune response to attack the cells producing the matrix or attack the matrix itself. The extracellular matrix of a biofilm community exists, at least in part, to act as an immunoavoidance mechanism. Furthermore, in many cases, the matrix material is constantly being produced and sloughing off into the environment.

Polymicrobial diseases: considerations for vaccine development

Although many infectious diseases are initiated by a single pathogen or virulence factor, others originate from or are attributed to a complex milieu of microorganisms. Examples of diseases associated with both polymicrobial and biofilm phenotypes include periodontal disease, otitis media, rhinosinusitis, ventilator-associated pneumonia, and chronic wound infections (Brogden *et al.*, 2005). These biofilm consortia of microorganisms typically coexist as combinations of highly structured communities of bacteria, viruses, protozoans, and fungi attached to biotic and environmental surfaces, where their architecture is facilitated by specific intermicrobial and host interactions (Bakaletz, 1995; Viale & Stefani, 2006; Kuramitsu *et al.*, 2007). Many of these interactions are mutually beneficial for both the host and the microorganism (e.g. the gastrointestinal and oral microbiota). However, microbial species population shifts and waning host immunity can allow colonization and subsequent infection by opportunistic pathogens that exploit unique niches in the polymicrobial environment (Stecher & Hardt, 2008). Despite the challenges of implementing polymicrobial vaccines, several have been attempted and proven successful, while others have yielded unexpected findings.

Traditionally, the guidelines for vaccine development for monomicrobial infections often rely heavily on molecular Koch's postulates, such that directing an immune response against a single virulence or colonization factor will provide protection against disease (Falkow, 1988). Although these rules have proven invaluable for vaccination against several diseases (e.g. *C. diphtheriae*), they do not adequately consider the pathogenesis of polymicrobial infections. It has been well documented that biofilm communities demonstrate a significantly different repertoire of gene and protein expression as compared with their planktonic counterparts (Dykes *et al.*, 2003; Waite *et al.*, 2006). However, little is known about the transcriptomic and proteomic profiles of multispecies biofilms assessed against monomicrobial communities. The pleiotropic effects of intermicrobial interactions on the individual disease-causing pathogens and the infected host are only now being appreciated. A recent study by Sibley *et al.* (2008) used a *Drosophila* polymicrobial disease model and luciferase reporter assay analyses to examine the effects of human oropharyngeal commensal isolates in coculture with *Pseudomonas aeruginosa* during infection. The results from this study demonstrated that the virulence of *P. aeruginosa* could be substantially enhanced or reduced dependent on the presence of a coinfecting microorganism that was nonpathogenic independently. Even more surprising was the modulation of host antimicrobial and innate immunity genes due specifically to polymicrobial vs. monomicrobial infection. These altered microbial and host profiles are likely due to the unique physical interactions and chemical signaling events that occur during the development of polymicrobial communities (Hogan *et al.*, 2004; Bamford *et al.*, 2009). Therefore, antigenic targets should be screened *in vivo*, via biologically relevant routes of infection or colonization, to ensure that immunogenic proteins of interest are expressed during infection and in the context of a polymicrobial environment as has been described previously (Rollenhagen *et al.*, 2004; Brady *et al.*, 2006; Hagan & Mobley, 2007).

The impact of the polymicrobial nature of a disease regarding colonization and infection should also be considered during vaccine development. A disease must first be classified as truly polymicrobial based on sufficient data from clinical studies and epidemiological records. Important criteria regarding the temporal shifts, composition, abundance, and consistency of microorganisms present throughout the entire course of the disease, from

colonization to fulminant infection, should be considered (Roberts, 1989; Tarsia *et al.*, 2007). One must also distinguish contaminating microorganisms (pathogens or commensals) from those that initiate and propagate infection. If a disease is considered to be of a polymicrobial nature, a vaccine composed of a multivalent cocktail of antigenic proteins from all microorganisms involved in disease pathology may be warranted. Although seemingly trivial, these criteria are crucial to understanding the pathogenesis of and developing effective vaccines for multimicrobial diseases.

Polymicrobial infections represent a significant complexity in vaccine development. Two (or more) microorganisms may act synergistically or antagonistically to mediate disease while either in isolation is differentially virulent or benign (Carlson, 1983; Diebel *et al.*, 1999). Even if a vaccination attempt successfully negates a necessary virulence factor for one pathogen (i.e. a toxin), virulence could be complemented *in trans* by another factor produced by a neighboring species in the polymicrobial community. In addition, the eradication of one species from the polymicrobial community may be insufficient at reducing overall disease, as another organism present may fill in the niche left behind. Alternately, a vaccination attempt targeting a virulence factor (i.e. an adhesin) for one pathogen may successfully target and eradicate a secondary pathogen within the polymicrobial infection.

Modulation of a microorganism's pathogenicity by the polymicrobial community has important implications for vaccine development as studies for *S. aureus* suggest. A formidable nosocomial pathogen, *S. aureus* can be isolated as the single etiologic agent in a multitude of diseases (e.g. sepsis, lower respiratory tract infections, skin infections, and others) or among a polymicrobial community in the same disease types. Polymicrobial infections complicate approximately 27% of nosocomial *Candida albicans* bloodstream infections; among these, *S. aureus* is the third most common coinfecting microorganism (Klotz *et al.*, 2007). As microbial biofilms on indwelling medical devices act as a potential nidus for planktonic release and onset of sepsis, observations of enhanced biofilm formation and differential matrix composition for *S. aureus* in coculture with *C. albicans* suggest that polymicrobial interactions may facilitate *S. aureus* colonization and disease onset (Harriott & Noverr, 2009). The synergistic action of *C. albicans* and *S. aureus* has also been implicated in the increased mortality of mice infected with *S. aureus* strains producing the toxic shock toxin (Carlson, 1983). Indeed, vaccination against *C. albicans* using the candidal adhesion Als3P can provide cross-kingdom protection against *C. albicans* and *S. aureus*, and has positive implications for controlling diseases mediated by coinfection of these microorganisms (Spellberg *et al.*, 2008).

In summary, polymicrobial infections require ecological and physiological characterization to determine interactomes and changes in target expression based on community characteristics. Therefore, vaccine design for polymicrobial infections should adequately consider the consortia of microorganisms responsible for disease, potential inter microbial interactions resulting in the modulation of *in vivo* expressed antigens, and the strategic elimination of microorganisms that enhance or contribute to pathogenesis. Future strategies may be to target vaccination against seemingly nonpathogenic organisms that facilitate increased pathogenicity and colonization of virulent microorganisms. Of course, vaccination against 'commensals' may have deleterious immunological and microbiological consequences in the host and will have to be tested rigorously before utilization.

Considerations for future vaccines: lessons learned from *S. aureus*

Effective vaccines are available today for many previously problematic bacterial infections, such as the triple vaccine against *C. diphtheriae*, *C. tetani*, *Bordetella pertussis* (Pichichero *et al.*, 2006), *N. meningitidis* (Trotter *et al.*, 2008), and *S. pneumoniae* (Bernatoniene & Finn, 2005). The infections targeted by these vaccines are all mediated by one or a few virulence factors, which, when blocked or otherwise neutralized, prevents pathogenesis. Alternatively, other microorganisms have presented a significant challenge in vaccine development due to a complex disease process and the presence and expression patterns of their respective virulence factors. One such example is *S. aureus*. This pathogenic species is able to cause a host of different types of infections that are either planktonic (e.g. sepsis and pneumonia), biofilm mediated (e.g. osteomyelitis, endocarditis, chronic skin infections, indwelling medical device infections, chronic rhinosinusitis, dental implantitis, and endophthalmitis), or a combination of both modes of growth (e.g. abscess).

Staphylococcus aureus is able to accomplish this array of infections by possessing nearly 70 virulence factors, each with infectious mode-of-growth and time-specific expression patterns. Therefore, the search for a single candidate antigen effective in all these cases has hindered *S. aureus* vaccine development. Additionally, the ability of these vaccines to provide protection against multiple modes of growth, including both planktonic and biofilm infection, has not been addressed adequately. While the suggestion of a prophylactic vaccine against the biofilm mode of growth seems counterintuitive, details emerging about *S. aureus* pathogenicity and modulation of the host immune response support this concept. In addition to the multitude of innate immunity evasion tactics (e.g. inhibition of neutrophil

chemotaxis, inactivation of complement factors, depletion of leukocyte levels, and inhibition of phagocytosis) (Foster, 2005), *in vitro* and *in vivo* studies indicate that *S. aureus* factors direct the host response toward a beneficial one for the pathogen. *In vitro* cytokine analyses demonstrate a robust Th1 immune response elicited against *S. aureus*: staphylococcal enterotoxin B induces IL-2 and IFN-γ (Assenmacher *et al.*, 1998), staphylococcal enterotoxin B induces THF-α and MIP-1β (Dauwalder *et al.*, 2006), and whole-cell *S. aureus* induces IL-12 p70 and IL-18 (Buzas *et al.*, 2004). Studies in a murine model of prosthetic implant infected with *S. aureus* found upregulation of Th1 cytokines (IL-2, IL-12 p70, and TNF-α) and Th17 cytokines (IL-6 and IL-17) at days 7 and 28 postinfection and increased levels of IgG2b (the dominant Th1-dependent iso-subtype) compared with IgG1 (a Th-2 dependent iso-subtype) in the serum at day 7 postinfection (R. Prabhakara & M. E. Shirtliff, unpublished data). These studies indicate that *S. aureus* elicits a prolonged Th1 response, where the proinflammatory defenses are thwarted by the microbial virulence factors and cause significant damage to the host tissue, and subverts a Th2 humoral response; these skewed immune responses allow the planktonic *S. aureus* to elude clearance by the immune system as the microorganism colonizes the damaged host tissue and forms a biofilm. Therefore, in order to encompass all aspects of staphylococcal virulence in vaccine development, one must also include an emphasis on biofilms.

Antigen selection: the next generation

In order to correctly select appropriate antigens that will be effective in preventing the establishment of a microbial infection, it is necessary to take into account the planktonic and biofilm modes of growth. Microbial biofilms present a unique challenge to researchers seeking to develop vaccines against microorganisms whose infectivity depends, wholly or in part, on this growth modality. Success cannot be achieved by ignoring the fundamental principle of microbial biofilms: *biofilm-resident bacterial cells exhibit a phenotype that is distinct, and in some cases, almost unrecognizable, compared with that of taxonomically identical cells growing planktonically* (Beenken *et al.*, 2004; O'May *et al.*, 2009). Thus, both the planktonic and the biofilm phenotype and its implications for antigen expression must be taken into account during the selection of antigens to be included in a vaccine. While the search for a single antigen that provides multimodal protection may prove successful, it seems more likely that a multicomponent vaccine will be necessary. This is the first criterion for an effective broad-range vaccine.

The second is to ensure that the selected antigens are expressed in all relevant strains of the pathogen targeted by the vaccine. The genetic variation of surface-expressed proteins between strains also raises a difficulty. Just such a problem (Thompson *et al.*, 2003; Dyet & Martin, 2005) as well as the structural homology of the polysaccharide capsule with the polysialylated form of the neural cell adhesion molecule (Finne *et al.*, 1983) has held up the development of a broad-range vaccine against type B *N. meningitidis*, although clinical trials have begun on vaccines developed by reverse vaccinology and other strategies (Granoff, 2010; Sadarangani & Pollard, 2010). For this reason, it is vital to test vaccine efficacy against as large a number of strains as is realistically feasible.

The third principle is to ensure that the candidate antigens are expressed *in vivo* throughout the infection cycle in the multiple types of infection (e.g. sepsis vs. indwelling medical device infection) for which the pathogen is the identified etiological agent. Once again, like the multiple modes of growth, this protection will most likely need to be accomplished by a multivalent vaccine.

The fourth principle of antigen selection is that either (1) the selected antigen, or (2) the sum of all antigens included in a multicomponent vaccine, must be expressed throughout the infecting microbial population. This is particularly the case when prevention of biofilm-type infections is the goal. Biofilm communities are inherently complex systems, usually existing in close proximity to a surface. This complexity arises from a number of factors. First, distinct physicochemical gradients are found within microbial biofilm communities. In most cases, organic compounds, oxygen, or water enter the biofilm from the surrounding bulk fluid and diffuse through the matrix to the depths closer to the surface. Bacteria resident within a biofilm consume these compounds at varying rates, resulting in differential availability of nutrients, dependent on the location of a particular cell within the community. This effect has been observed experimentally in the case of oxygen tension (de Beer *et al.*, 1994). The situation is further complicated by very low metabolic levels and radically downregulated rates of cell division of the deeply entrenched microorganisms (Brown *et al.*, 1988), including totally nondividing 'persister' cells (Harrison *et al.*, 2005; Lewis, 2008). This lowered growth rate is partially responsible for the increased recalcitrance to antimicrobials exhibited by biofilm-embedded bacteria (Gilbert *et al.*, 2002). The end result of this is that cells in different areas of the biofilm exhibit spatial phenotypic heterogeneity, i.e. an antigen expressed by cells in a relatively nutrient-rich area of the community may not be expressed by other cells under less favorable growth conditions. A study by Brady *et al.* (2006) on *S. aureus* investigated the ability of polyclonal IgG raised in rabbits against antigens, shown in an earlier work by the same authors to be expressed in *S. aureus* biofilm *in vivo*, to visualize *S. aureus* biofilm communities grown in an *in vitro*

flow reactor (Brady *et al.*, 2007). Data suggested that although each of the four antigens was expressed within *S. aureus* biofilm communities, none of them was expressed homogenously throughout the biofilm. Instead, differing expression patterns were observed for each antigen. Hence, inclusion of any one antigen in a monovalent vaccine would likely mean that only a fraction of the biofilm would be targeted and the biofilm would likely survive and the infection would persist. It follows that a multivalent vaccine is essential when prevention of biofilm-type infection is the goal.

Finally, the antigens selected for a biofilm vaccine must be immunologically relevant, meaning that they must be cell-surface proteins that are visible to the humoral immune system and not obscured by the biofilm matrix. Furthermore, each component must be capable of not only eliciting a strong humoral immune response in the host, but a correct response. In some cases, microbial clearance can be promoted by either an inflammatory response (Th1 and/or Th17) or an anti-inflammatory response (Th2 and/or Treg) that can be disease mode, species, or even microbial strain specific. Once again, multivalent vaccines seem to be required to accomplish this principle.

Brady and colleagues used these criteria to select four protein antigens that were demonstrably expressed during *S. aureus* biofilm growth *in vitro*, cell-surface associated, and immunogenic in the rabbit model of osteomyelitis (Mader & Shirtliff, 1999; Brady *et al.*, 2007). Singly, combined with the TiterMax^{TM} adjuvant comprised of squalene, sorbitan monooleate 80, and a synthetic block copolymer CRL8941, these antigens were unable to provide protection against *S. aureus* osteomyelitis in the rabbit model. However, when used together as a prophylactic quadrivalent vaccine (75 μg of each protein administered subcutaneously; one booster 14 days later; both using the TiterMax^{TM} adjuvant) and combined with postinfection vancomycin treatment (5 mg kg^{-1} twice daily for 10 days) to eliminate planktonic bacteria residing within the bone, eight of nine animals cleared the infection completely. Furthermore, there were significant reductions in radiological and clinical signs of infection in the treated vs. the untreated groups (Brady *et al.*, in press). Research now being conducted is seeking to include *S. aureus* surface proteins expressed during planktonic growth in order to remove the need for concurrent vancomycin administration.

The unique physiology and properties of biofilm must be taken into account when selecting antigens for inclusion in any vaccine intended to be effective against these communities. Biofilm-type infections can no longer be regarded as merely 'bacteria embedded within slime'. Biofilm-resident microorganisms are distinct from their free-living counterparts and present unique challenges to anyone seeking to develop novel prophylactic therapeutics.

Conclusions

Vaccine development has primarily focused on the pathogenesis of a single microorganism based on its virulence and immunoavoidance factors and the directed host response to the monomicrobial infection. However, greater appreciation of the fact that many infectious diseases result and persist due to the polymicrobial nature and biofilm maturation of bacteria is challenging many perceptions on vaccine design. Current recombinant vaccines targeting a single or a few bacterial proteins possess the benefits of easy manufacture, no risk of disease from reversion back to a virulent form, and few adverse effects from inflammatory induction compared with whole-cell vaccines. Recombinant vaccine usage does come with the loss of antigen diversity and robust humoral response due to the innate response activation that is provided from vaccination with whole cells. As such, redundancy in bacterial proteins expressed during infection, for example adhesins, subverts responses activated by monovalent vaccines and provides incomplete protection. Antigenic variation has also compelled reassessment of vaccine design due to the observation that in vaccinated individuals the diseases targeted by current clinical vaccines, for example *S. pneumoniae* 7-valent, shift toward ones actuated by previously scarce and inconsequential bacterial variants that are not represented in the vaccine (Eskola *et al.*, 2001). Multivalent strategies have come to the forefront in vaccine development in hopes to provide antigenic diversity and sufficient vaccine efficacy, but some clinical trials with multivalent vaccines fail to transition into a later phase, due to the incomplete coverage against disease that is observed.

Staphylococcus aureus-mediated diseases highlight the key properties of the pathogen that are challenges to current vaccine strategies and not appropriately addressed during most vaccine development efforts, including polymicrobial infection, biofilm maturation, and host carrier status. Vaccines targeting *S. aureus* adherence factors could be ineffective against diseases where coinfecting microorganisms contribute virulence factors in *trans* and negate the activity of the *S. aureus* factors, for example hypothetical control of *S. aureus* adherence by the *B. pertussis* secreted proteins during coinfection that mimics *in vitro* findings (Tuomanen, 1986). Once *S. aureus* colonization is successful and *S. aureus* immunoavoidance factors obstruct the innate immune response, *S. aureus* may grow and persist as a biofilm community encapsulated in a polysaccharide matrix. Compounding the problem is that this timed up- and downregulated expression of virulence factors is not only growth phase dependent but also disease specific.

The biofilm phenotype further conceals *S. aureus* from the immune system due to the downregulated expression of factors that mediate initial infection and encapsulation in polysaccharide that masks surface-associated proteins from

immune recognition. Analysis of the mature *S. aureus* biofilm indicates that there is great heterogeneity in protein expression throughout the biofilm community, with protein expression present in some microcolonies and completely absent in others. As such, a vaccine that targeted these proteins would be ineffective at eliciting an opsonization response to clear *S. aureus*.

Another consideration for vaccine development is the expression of virulence factors that antagonize the immune response, inducing inflammation and tissue damage, where further bacterial colonization can occur; other factors that target and inactivate host immunoglobulins also pose significant problems. Knowledge of the specific immune responses activated by the bacteria and whether that response assists bacterial colonization and persistence will allow the development of vaccines that can modulate the immune response, using adjuvants or extrinsic bacterial components, which skew toward appropriate immunity.

A final consideration for vaccine development is *S. aureus* carriage in humans. Analysis of sera from healthy carriers establishes the circulation of anti-*S. aureus* immunoglobulins, indicating that this response is insufficient to prevent colonization and persistence. Vaccine strategies using antigens targeted by those immunoglobulins will probably elicit a response that is not completely protective. Therefore, screening for and removal of those antigens before protection studies may be advisable. Overall, these properties are critical to understanding how the immune response is ineffective at bacterial clearance. Further evaluation of these features will establish optimal antigenic candidates, including protein factors specific for disease and those not concealed from the immune system that should be established as prerequisites for *S. aureus* and other bacterial vaccines.

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Infection Prevention and Control in Deployed Military Medical Treatment Facilities

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Abstract: Infections have complicated the care of combat casualties throughout history and were at one time considered part of the natural history of combat trauma. Personnel who survived to reach medical care were expected to develop and possibly succumb to infections during their care in military hospitals. Initial care of war wounds continues to focus on rapid surgical care with debridement and irrigation, aimed at preventing local infection and sepsis with bacteria from the environment (e.g., clostridial gangrene) or the casualty's own flora. Over the past 150 years, with the revelation that pathogens can be spread from patient to patient and from healthcare providers to patients (including via unwashed hands of healthcare workers, the hospital environment and fomites), a focus on infection prevention and control aimed at decreasing transmission of pathogens and prevention of these infections has developed. Infections associated with combat-related injuries in the recent operations in Iraq and Afghanistan have predominantly been secondary to multidrug-resistant pathogens, likely acquired within the military healthcare system. These healthcare-associated infections seem to originate throughout the system, from deployed medical treatment facilities through the chain of care outside of the combat zone. Emphasis on infection prevention and control, including hand hygiene, isolation, cohorting, and antibiotic control measures, in deployed medical treatment facilities is essential to reducing these healthcare-associated infections. This review was produced to support the *Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011* Update contained in this supplement of *Journal of Trauma.*

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Modern technology, doctrine, and training have resulted in improved survival of personnel injured on the battlefield, including those with severe combat-related injuries. In addition to body armor, immediate life-saving techniques such as tourniquet and hemostatic bandage use, field cricothyroidotomy, and rapid evacuation from the battlefield using aircraft, medical treatment facilities (MTFs) are positioned throughout the combat zone to allow rapid surgical stabilization and ultimately transportation of the injured back to their home nations. Care of the wounded is initiated at the point of injury by self-aid, buddy aid, combat lifesavers, and/or combat medics/corpsmen. Wounded personnel are then transported to increasingly higher levels of care (Table 1) until they reach definitive and rehabilitative care back in their home nation. Transportation of patients between levels (roles) of care is dependent on combat activities, location, transportation assets, weather, terrain, and military control of ground or air. In the current conflicts, most casualties are transported from point of injury to Role 2 or 3 care via helicopters. Once stabilized, most patients will then need to be transported again to a Role 3 hospital from which aeromedical evacuation out of the combat zone is possible. For wounded US personnel in the current conflicts, this entails long distance aeromedical evacuation to Germany (Role 4, Landstuhl) and then to continental US military medical centers (Role 5), most commonly by C-17 aircraft. This complex chain of care, by its nature, requires multiple physical and care management handoffs over a short period of time, typically 3 days to 7 days from point of injury to care in a medical facility in the patient's home country.

The focus of this review is care within combat zone MTFs. This care is often provided in less than ideal environmental conditions by staff who, by the nature of military deployments, are transient members of the MTF. Challenges to provision of care have been previously described and include high personnel turnover rates, provision of care to local nationals and non-US personnel, physical structure of MTFs, environmental conditions, and the logistical support chain (Table 2).[1–3] These challenges all make the effective practice of infection prevention and control difficult in deployed MTFs. In this article, we review the history and challenges of healthcare-associated infections (HAIs) in deployed MTFs as they pertain to caring for combat-injured

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TABLE 1. Care and Resources Available Across the Various Strata of Medical Support for Patients Injured in Combat Operations

Designation*	MTF or Site of Care	Care Provided/Resources
Role 1/Level I	Point-of-injury (field care)	Self-aid, buddy aid, combat lifesaver, combat medic/corpsman care
	MTF: battalion aid station (US Army), shock trauma platoon (USMC)	Physician/physician assistant care, no patient holding capacity
Role 2/Level II	MTF: medical company (includes forward support medical company, main support medical company, and area support medical company, US Army), expeditionary medical support (USAF)	72-h patient holding capacity, basic blood transfusion, radiography and laboratory support
Role 2b/Level IIb	MTF supplemented with surgical assets: forward surgical team (US Army), mobile field surgical team (USAF), forward resuscitative surgical system (USMC)	Forward resuscitative and stabilization surgical care
Role 3/Level III	MTF: combat support hospital (US Army), Air Force theater hospital, (USAF), casualty receiving ships (USN)	Full inpatient capacity with intensive care units and operating rooms
Role 4/Level IV	MTF: regional hospital (Landstuhl Regional Medical Center, Germany) or USNS hospital ships (USN), typically outside of the combat zone	General and specialized inpatient medical and surgical care
Role 5/Level V	MTF: military care facilities within United States, typically tertiary care medical centers	General and specialized inpatient medical and surgical care, rehabilitative care

MTF, medical treatment facility; USMC, US Marine Corps; USAF, US Air Force.
* Level or echelon are considered equivalent terms to role.

TABLE 2. Challenges in Deployed Medical Treatment Facilities That Potentially Impact Infection Prevention and Control Efforts

Challenge	Impact or Potential Impact
High personnel turnover rate	Limit institutional memory. Hospital personnel, including leadership, change at rates higher then permanent US facilities influencing any/all long-term programs.
Provision of care to local nationals and non-US personnel	Prolonged hospital stays. Options to transfer these patients to lower levels of care once stabilized may be limited by resources available in the community and risks to the individual patients in the local community.
Physical structure of medical treatment facilities	Use of preexisting structures not designed as modern hospitals results in space constraints including crowding, limited numbers of private rooms, and less than ideal configurations for optimizing infection control practice. Deployable structures (e.g., tentage) may make infection control challenging.
Environmental	Extremes of hot or cold temperatures, rain, snow, dust, and dust storms challenge design and operation of deployed facilities. Hostile environment add physical and operation challenges.
Logistical support chain	Receipt of supplies via a long supply chain which passes through hostile territory can result in temporary shortages of items or substitution with available but not identical items.

Adapted with permission from *J Trauma*.[1]

personnel. We also review the history and current practice strategies available to decrease or prevent these infections.

BACTERIOLOGY OF WAR WOUNDS

Before the use of rapid surgical management, early debridement and irrigation, and adjunctive postinjury systemic antimicrobials, most infections associated with combat-related injuries occurred soon after wounding and were secondary to bacteria that contaminated wounds at the point of injury.[4] These included *Clostridium perfringens*, the cause of gangrene, from the soil, and aerobic gram-positive cocci of the skin, including *Streptococcus pyogenes* and *Staphylococcus aureus*. If wounding resulted in the breaching of the gastrointestinal (GI) tract, the bacteria that constitute the GI flora could also contaminate wounds. Patients who survived past this initial insult were subsequently at risk for HAIs in hospitals established in, and outside of, the combat zone. The introduction of antimicrobials to help ameliorate these infections has been associated with the selection of bacterial pathogens resistant to these antimicrobials.

Natural History

In World War I, Sir Arthur Fleming described three stages of wound bacterial flora/infection. The first stage (days 1–7) is characterized by foul-smelling, watery discharge and predominantly sporulating anaerobes (likely clostridia) and streptococci. The second stage (days 8–20) is characterized by purulence and pyogenic cocci. The third stage (>20 days from wounding) is oftentimes identified with simple infection by streptococci or staphylococci.[5] This was verified and further defined by studies of war wound bacteriology in World War II. Studies during that war found that although pyogenic organisms (*S. pyogenes* and *S. aureus*) were only rarely (5–6%) recovered from wounds at hospital admission, those bacteria were common causes of wound infection, infecting >50% of wounds, after 1 week, and increased up to 70% to 90% thereafter.[6]

Influence of Antimicrobials

With the institution of topical and later systemic postinjury antimicrobial therapy (prophylaxis) during and after World War II (in addition to early surgical debridement and irrigation), bacteria resistant to these antimicrobials, espe-

cially gram-negative bacteria, have filled the niche previously occupied by soil anaerobes and skin streptococci and staphylococci. The postinjury use of penicillin and streptomycin during the Korean War was associated with 83% and 85% resistance, respectively to these antimicrobials, in bacteria recovered from infections diagnosed upon transfer to the US military hospital in Japan.[7] A study conducted during the Vietnam War documented a transition of wound bacteria from those typically found on skin to predominantly gram-negative bacteria, most commonly *Pseudomonas aeruginosa*, by day 5 after injury.[8]

Multidrug-Resistant Bacteria Colonization and Infection of Wounds

Numerous reports have documented the epidemiology of colonization and infections associated with the recent conflicts in Iraq and Afghanistan.[9–12] Multidrug-resistant (MDR) gram-negative bacilli, including *Acinetobacter baumannii-calcoaceticus* complex, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (e.g., *Escherichia coli* and *Klebsiella pneumoniae*), *P. aeruginosa*, and methicillin-resistant *S. aureus* (MRSA), have most commonly been reported as the cause of these infections.[12–14] Over the past decade, carbapenem susceptibility has dramatically declined in *Acinetobacter* isolates recovered from those personnel injured in combat in Iraq and Afghanistan.[12,15] Accumulated data support nosocomial spread of these MDR bacteria within deployed MTFs and likely throughout the military healthcare system (Fig. 1). With the exception of MRSA, it does not appear that US personnel are colonized with these bacteria before injury. Colonization with community-associated MRSA has been documented in healthy military personnel and is a potential source of later infection.[16–18] Preinjury colonization by resistant gram-negative bacteria in military personnel, specifically *Acinetobacter*, has not been found in small studies of deployed and never (pre-) deployed troops.[19–21] MDR bacteria have also not been found contaminating wounds at the time of admission to these deployed facilities.[22] Introduction of resistant bacteria into deployed MTFs through care provided to host nation and other non-US patients is a concern and likely source of colonization leading to later infection of our combat-injured personnel. Studies conducted in deployed MTFs have found associations between MDR bacteria and host nation patients as well as associations between duration of host nation patient intensive care unit stay.[23,24] Two studies conducted to specifically examine the possibility that local nationals were a source of MDR pathogens documented MDR colonization or infection of both Iraqi[25] and Afghan[26] patients around the time of admission to US military MTFs.

Globally, reports of the spread of ESBL organisms and more recently, carbapenem-resistant organism, like the New Delhi Metallo-β-lactamase-1 strains originating in the Indian subcontinent, have raised grave concerns of the expansion of resistance among gram-negative bacteria and spread of these MDR bacteria outside of the healthcare setting and into the community at large.[27] Indeed, a New Delhi Metallo-β-lactamase-1 strain has been recently recovered at the US military Role 3 hospital in Bagram, Afghanistan, in an Afghan patient admitted with burn injuries. Asymptomatic carriage in the GI tract by healthy persons is also a potential source of MDR pathogens. A recent study of asymptomatic travelers from Sweden found GI tract colonization with ESBL bacteria in an unexpectedly large number (24%).[28]

HAIS IN MILITARY HOSPITALS

In the late 1700s and early 1800s, hospitals were known for their malodorous stench from infected wounds and dead bodies. Wounds from both trauma and surgery were all expected to become purulent. The production of pus was considered an essential part of the healing process. This idea of "laudable pus" had been around since the time of Galen

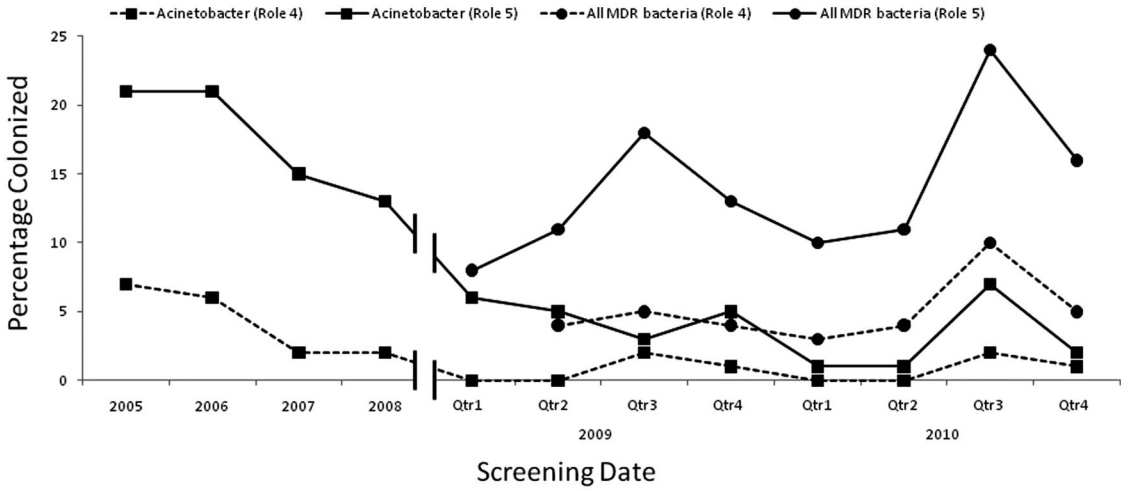

Figure 1. Colonization of injured US personnel upon arrival to Landstuhl Regional Medical Center (Role 4) from the combat zone and at three continental US medical centers (Role 5; Brooke Army Medical Center, National Naval Medical Center, and Walter Reed Army Medical Center) after transportation from Germany. Note: admitted personnel were only screening for *Acinetobacter* carriage from 2005 to 2008. Thereafter, admitted personnel were screened for all multidrug-resistant (MDR) bacteria.

(circa 130–200 AD).[29] Hospitals around the turn of the 18th century commonly had open wards with large beds that were occupied by multiple patients.[30] Bandages were reused, and the wounds of multiple patients were "cleaned" with the same sponge and water. HAIs have been recognized for >150 years. Described as "hospital infections", "added infections", and more recently, "nosocomial infections", Sir James Simpson used the term "hospitalism" in his 1867 publication.[31] Detailing the serious infections that plagued hospitalized patients of the time, Simpson reported data comparing the mortality in hospitalized and nonhospitalized patients. An example of these data is his report of 41% mortality following amputations performed in hospitals versus a noted 11% mortality with the same procedure performed in "country practice". During the American Civil War, most injured personnel who survived to hospital care died of infection, including tetanus, hospital gangrene, erysipelas, and pyemia.[32] Hospital gangrene and erysipelas were recognized at that time as contagious, and recommendations were made for cleanliness, ventilation, and against overcrowding. Both hospital gangrene and erysipelas are now postulated to be secondary to streptococcal infection.

In 1940, Miles et al.[33] described the epidemiology of microbiology of war wounds in hospitalized patients. Their description of "hospital infection—infection of the tissues with pathogenic microbes derived from the hospital environment" was supported by studies of serial wound cultures that documented changes in wound colonization/infection over time.[33,34] They identified colonization of hospital personnel with *S. aureus* in the nose and *S. pyogenes* in the nose and throat as likely sources of hospital wound infections. They also showed that wound pathogens (chiefly staphylococci and streptococci) could be found in the air of wards full of wounded soldiers, which they postulated were from cleaning, changing sheets, and wound care (dressing changes).[35] In addition to the hospital air, they identified fingers, instruments, dressings, baths, bed-pans, and urine bottles as likely sources of hospital infection.

RESPONSE TO HEALTHCARE-ASSOCIATED INFECIONS: HISTORY OF INFECTION PREVENTION AND CONTROL PRACTICES

Hand Hygiene

Although Hippocrates provided comment on the proper length of a surgeon's fingernails, neither too long nor too short,[36] it was Ignaz Semmelweis (1818–1865) who is credited with proving a direct connection between hand hygiene and HAIs. After noting the large difference in mortality rates of women dying from puerperal sepsis when delivered by physicians and medical students compared with midwives, Semmelweis deduced this might be because the groups differed in that the physicians and medical students performed the autopsies on the women who died of this complication.[31] Introduction (and enforcement) of hand cleansing with a hypochlorite solution (chloride of lime) after performing autopsies dramatically decreased mortality from puerperal sepsis in women delivered by physicians and medical students, comparable to the rate of midwives. Although the importance of hand hygiene became accepted before his death in 1865, strict adherence to hand hygiene remains a difficult goal to achieve even in modern hospitals in the 21st century.

Environment (Hospital) Hygiene/Sanitation/ Outcome Data Monitoring

Although not a believer in the germ theory, or that infection could be passed on the hands of healthcare providers, Florence Nightingale is held in the greatest esteem by the infection prevention and control community for her efforts in both hospital hygiene/sanitation reform and meticulous record keeping and application of statistics to support interventions. Sent by the British Army to Crimea in 1854, Nightingale's work to improve sanitation at the Scutari Hospital led to a drop in the hospital's mortality rate from 42% to 2%, between February and June 1855. This included environmental cleaning, provision of adequate food (i.e., improving patient nutrition), clothing, and bedding, and insistence on the maintenance of nursing staff personal hygiene.[31] She is quoted as saying, "Every nurse ought to be careful to wash her hands very frequently during the day. If her face too, so much the better".[31] Nightingale dedicated her life to sanitary reforms in the British military and United Kingdom. AQ: 4

The US Sanitary Commission was established in 1861, at the start of the American Civil War, to improve medical conditions within the military hospitals of the time.[32] It was recognized by that time that hospital cleanliness was necessary to allow recovery and wound healing. In addition to trying to maintain high standards of cleanliness/sanitation/hygiene, the use of bromide spraying into the air to stop erysipelas outbreaks was employed. After major outbreaks of hospital gangrene in 1862 to 1864, use of immediate patient isolation and basic sanitary precautions (dedicated patient sponge, toiletry items, and eating utensils) resulted in no further outbreaks of this infectious disease.[32] Use of individual patient sponges and basic sanitary conditions were suggested to decrease the incidence of pyemia (wound sepsis). Despite these efforts by the Sanitary Commission, it is interesting to note that surgeons during the American Civil War did not regularly wash their hands or surgical instruments.

Antisepsis and Asepsis

Joseph Lister (1827–1912) advanced the idea of antisepsis to surgery in 1867.[30] Supported by the discoveries of Louis Pasteur (in the 1850s–1860s) that germs (bacteria) were the cause of putrification (pus production), Lister promoted the use of carbolic acid solutions to improve surgical safety. During the American Civil War, three studies conducted using antiseptics (bromide, turpentine, and nitric acid) showed reduction of mortality from hospital gangrene. Specifically, one study reported <3% mortality in 308 patients treated with bromide for hospital gangrene (compared with 43% mortality in 30 untreated patients).[37] Before the Listerian era, surgical instruments were not even routinely cleaned, often simply wiped off between uses.[30] Suture was often carried in the surgeon's pocket. Antiseptic surgery became virtually universal between 1870 and 1890. Heat

sterilization of surgical instruments was introduced by Ernst von Bergmann in 1891.[29] In 1915, Keen reported, "Instead of hospitals reeking with pus and emptied by death, … we have hospitals of immaculate whiteness and emptied by quick recovery."[38]

Surgical Attire and Personal Protective Equipment

Sterile surgical caps and gowns were introduced in 1883 by Neuber and masks in 1897 by Mikulicz.[29] Gloves, initially used to protect the surgical nurse's hands from the antiseptic chemicals used in surgery, were adopted around the turn of the century (1890s–1900s) when it was noted that their use was also associated with lower rates of postsurgical infections (Fig. 2).[39] To interrupt the spread of infection among the war wounded, Miles et al.[35] espoused use of masks, dressing of wounds with clean dry hands and using sterile instruments, removal of dressings and plasters with minimum disturbance, and care of the hospital environment to minimize dust and disinfect key surfaces (e.g., baths). McKissock et al.[40] reduced infections in head wounds from 30% to 2% with use of aseptic dressing changes and dedication and disinfection of patient personal and care items.

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Isolation and Cohorting

Cohorting of patients with similar infectious processes was used during the American Civil War to prevent spread of disease such as erysipelas to other patients. Miles reported that the risk of infections associated with wounds was greatly reduced by the practice of antisepsis and asepsis and of the segregation of grossly infected cases.[33,34]

Mobile Surgical Hospitals and Deployed Research Laboratories

In World War I, Antoine Depage (1862–1925) helped advance combat wound management through reintroduction of debridement, use of delayed wound closure based on microbiology sampling, and organization of mobile surgical units.[41,42] Alexander Fleming performed microbiologic studies of the war wounded in laboratories associated with Depage's hospital. This idea of a deployed research cell to support the advancement of combat casualty care was used by the United States during the Vietnam War and most recently in Iraq and Afghanistan.

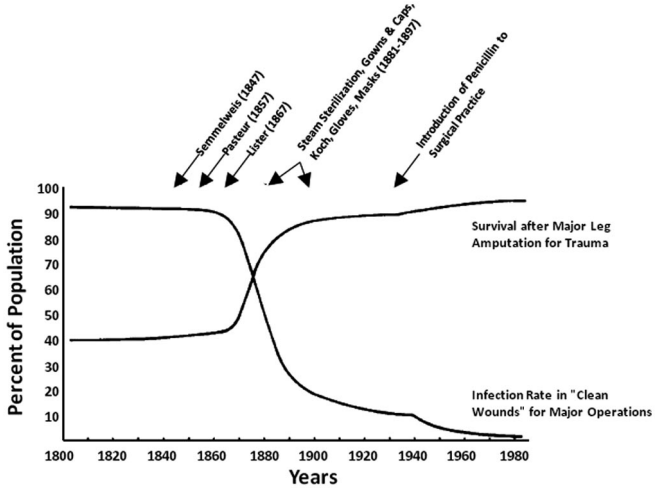

Figure 2. Impact of aseptic surgery and other infection prevention and control practices on postsurgical infections and survival. Reproduced with permission from *Ann Surg.*[30]

INFECTION PREVENTION AND CONTROL IN THE DEPLOYED SETTING

The effective practice of infection prevention and control in the deployed setting holds all the challenges that are present in fixed Western hospitals, but also must meet the unique challenges of the combat zone. The challenges unique to the deployed setting have been described in recent reviews, including in conjunction with specific combat zone reviews of infection control practice and challenges conducted in 2008 and 2009.[1–3] From these reviews, specific areas for improvement have been identified (Table 3).

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Emphasis on Infection Prevention and Control Basics

Success of an effective infection prevention and control program in a deployed hospital hinges on the same factors as in modern fixed facilities anywhere. These include emphasis by all personnel, education and reeducation of healthcare

TABLE 3. Specific Infection Control Areas Identified for Improvement in Deployed Hospitals and Recommendations

Area Identified for Improvement	Recommendations for Improvement
IC expertise	• Provide improved predeployment ICO training through use of AMEDD C&S short course or other established courses • Establish theater-level IC consultant • Use outside experts to assist via electronic (teleconferencing, email) and in-theater reviews • Require facilities to develop annual IC plans and summaries
Emphasis on basic IC measures	• Establish hand hygiene programs with command emphasis and compliance monitoring • Apply transmission-based (isolation) precautions when MDRO colonization or infection is suspected or proven • Use patient cohorting to separate short-term and long-term patients
Use of standardized procedures and guidelines	• Establish theater-level IC SOPs • Apply national (US) guidelines to prevent and treat HAIs • Monitor guideline compliance
Antimicrobial control	• Emplace antibiotic control programs • Use national (US) and other guidelines to limit duration and overuse of broad spectrum antibiotics • Continue to expand in-theater microbiology capabilities and establish antibiograms for individual facilities

IC, infection control; AMEDD C&S, Army Medical Department Center and School; MDRO, multidrug-resistant organisms; SOPs, standard operating procedures.
Adapted with permission from *J Trauma.*[1,3]

providers, and emphasis and oversight by the MTF leadership. Standard precautions should be used to prevent the transmission of pathogens from both recognized and unrecognized sources. The major component of standard precautions is hand hygiene (i.e., washing or cleansing hands before and after every patient interaction). Other components include the use of personal protective equipment (gloves, gowns, masks, and eye protection) when indicated. Although the importance of hand hygiene has been stressed for more than 100 years, maintaining high levels of compliance in even modern, well-funded Western hospitals has continually proven difficult.[43] In the deployed setting, with less than ideal facilities and sometimes limited resources, hand hygiene compliance is an even bigger challenge. With the recent emergence of waterless hand sanitizers, lack of or limited availability of water should no longer prevent the performance of hand hygiene. As with all infection prevention and control, the key to success in promotion of this essential keystone is emphasis, education, and leadership. Hand hygiene programs with compliance monitoring should be established in all deployed MTFs.

Another fundamental infection prevention and control tenet, use of transmission-based (isolation) precautions, must also be used in all deployed MTFs. Using contact, droplet, and airborne precautions in the deployed setting can pose a much greater challenge than that of basic hand hygiene (Table 4). Patient segregation may be limited by the size and design of the buildings, portable hospital modules, or tentage T4

TABLE 4. Isolation Precautions to Prevent Transmission of Infections in Deployed Hospitals

Isolation Category	Patient Placement	Provider PPE
Contact—infection transmitted by direct contact with the patient or indirect contact with environmental surfaces or patient care items. Examples include MDR bacteria and diarrheal disease	Best: private room	Best: disposable gown and gloves for all interactions that may involve contact with the patient or potentially contaminated areas in the patient's environment. Changing PPE and hand hygiene between patients
	Good: bed separated from other patients by >3 feet	Good: gloves with removal and handwashing after each patient contact
Droplet—infection transmitted by droplets (can be generated by cough, sneeze, talking, or the performance of procedures). As these pathogens do not remain infectious over long distances special air handling and ventilation are not required. Transmitted via conjunctiva, nasal and oral mucosa. Examples include meningococcus, diphtheria, mumps, pertussis, influenza, and adenovirus	Best: private room	Best: surgical mask when entering room
	Good: cohort with other patients with same symptoms. Spatial separation of >3 feet with curtain between patient beds. If no curtains, consider keeping the patient 6–10 feet away from other patients	Good: surgical mask within 6–10 feet of the patient Note: Patient should wear surgical mask during transport. Request patients to cough/sneeze into tissue
Airborne—infection transmitted by airborne nuclei or small-particles of the size that can be deeply inspired. These particles can remain infective over time and distance (can be dispersed widely by air currents within a room or over a long distance). Examples include TB, varicella virus (chickenpox and disseminated shingles), smallpox, and rubeola (measles)	Best: private room with negative-air pressure, discharge of air to the outdoors or through high-efficiency filtration before recirculation. The door to the room must remain shut	Best: wear of N95 respirator at all time when in patient room or immediate environment. Personnel should be fit tested using the brand/model N95 respirator used at the facility
	Good: private room with a fan exhausting outward. The door to the room must remain shut	Good: wear of N95 respirator as above without fit testing
	Note: If no private room available, place patient as far as possible away from other patients in a well ventilated room with a physical barrier around the patient. Make sure patient is not near air intakes. Ideally, these patients should not be admitted to facilities without a negative pressure rooms. Consider housing them in private quarters outside the hospital and examining them outside in the sunlight	Note: Patient should wear surgical mask (not N95 respirator) during transport
Cohorting—when individual patient rooms are not available, patients with the same infection or presumed infection/colonization pattern can be housed in the same room or grouped in the same area of an open ward (if airborne pathogens are not suspected). Examples include influenza and varicella virus (chickenpox). In the deployed setting, this can be applied to patients with presumed MDR bacterial colonization based on duration of hospitalization. An arbitrary time of 72 h has been promoted by this group.	Use above based on expected pathogen(s)	Use of above based on expected pathogen(s)

PPE, personal protective equipment.
Modified with permission from *J Trauma*.[1]

used by any individual MTF. Lack of private rooms should not prevent the use of contact or droplet precautions. Physical barriers (e.g., empty beds) or markers (e.g., red duct tape delineation or construction cones on the floor) can be used to ensure adequate separation of patients. Use of airborne precautions in the deployed setting without properly engineered rooms poses the most difficult isolation challenge. Use of a private room with a strong fan pulling air to the outside is a potential work around within the MTF.[44] Establishing a patient care area outside the main MTF structure in a tent or isolated building/housing unit may provide more protection for other patients and staff.

As was done in the American Civil War, cohorting of patients presumed to have the same infection is a viable option during outbreaks (e.g., diarrhea, dysentery, and influenza). As described in previous articles, cohorting can also be used to separate patients at high risk for colonization with MDR pathogens from recent admissions unlikely to be carrying these bacteria. Therefore, it is suggested that newly admitted patients, especially those with open wounds, be separated (physically and by assigning designated nursing and other care team staff) from those patients who have been admitted for >72 hours.

The simple system described by Spaulding[45] in 1968 continues to underlie the practice of disinfecting and sterilizing hospital equipment and surfaces. Using this system, patient care and contact items are divided into critical, semicritical, and noncritical. Critical items include those that enter sterile tissue or the vasculature. These items should be purchased sterile or steam sterilized if possible. Semicritical items are those that come into contact with mucous membranes or nonintact skin. These items require high-level disinfection using US Food and Drug Administration (FDA) cleared chemical disinfectants. FDA-cleared high-level disinfectants include glutaraldehyde (e.g., Cidex), ortho-phthalaldehyde (e.g., Cidex OPA), hydrogen peroxide (e.g., Sporox), and peracetic acid (e.g., STERIS 20) based products. All other items fall under the category noncritical. These items can (and in the United States must) be cleaned with US Environmental Protection Agency (EPA) registered products. Low level EPA-registered products include quaternary ammonium, phenolic, and iodophor-based products, including Wexcide, Cavicide wipes, and Chlorox. Disinfection and sterilization should be performed based on national and professional society guidelines.[46]

Enhancing Deployment Infection Control Expertise

Because of the transient nature of staffing in deployed MTF, maintenance of an effective infection prevention and control program can be difficult. Personnel inexperience in the deployed setting and the lack of available trained infection control personnel can also pose challenges. With the large scale and duration of the US efforts in Iraq and Afghanistan, the need for infection control officers (ICOs) has been much greater than their availability. Reviews of deployed MTF in both 2008 and 2009 found this shortage of ICOs to be one of the most significant deficiencies.[1,3] Because of this identified issue, a 5-day infection control in the deployed setting course was established to provide basic training to personnel identified to serve as ICOs.[47] In the fall of 2010, assignment of an adequately trained ICO was made a US Army requirement for each deployed Role 3 location. In addition to the development of this short course, a universal standard operating procedure template was developed for use in the deployed MTF and supporting electronic resources produced.[3] These electronic resources include an Army Knowledge Online teleconsultation service that is monitored by US military infection control experts and internet resources (www.afids.org/links3.htm), which include links to key infection prevention and control and HAI management documents.

Antimicrobial Stewardship

Because of the association between the use of broad-spectrum antimicrobials and the development/selection of bacterial resistance, antimicrobial stewardship is also a key in decreasing colonization and infection with MDR bacteria. Limiting the use (and duration) of overly broad-spectrum antimicrobial agents can be encouraged by the use of treatment and prevention guidelines and through the availability of clinical microbiology. The timely availability of culture results, including antimicrobial susceptibility, is essential in tailoring antimicrobial usage (i.e., decreasing use of overly broad-spectrum empirical coverage) in deployed MTFs. Without the availability of clinical microbiology support, de-escalation of empirical broad-spectrum antimicrobial coverage is not possible. Use of guidelines and locally derived antibiograms are also important adjuncts to guide the appropriate use of antimicrobials. Stewardship programs can also include use of admission order overprints with specific antimicrobial selections, drug utilization evaluations, and antibiotic use approval programs.

Improvement of Epidemiology of Colonization and Infection

Wounded US military personnel are currently screened for colonization with MDR bacteria at admission to Role 4 and 5 MTFs (Fig. 1).[10] This testing provides data on the epidemiology of MDR colonization of wounded personnel as they arrive from the combat zone and after transportation to the continental US. The Multidrug-resistant Organism Repository and Surveillance Network was established in 2009 to further evaluate MDR bacteria and their associated epidemiology.[48,49] Both these programs can provide feedback to medical leaders in the combat zone on new and ongoing MDR threats.

RESEARCH GAPS

Many areas of research are greatly needed to further reduce the rates of infections in deployed hospitals. These include research into the epidemiology of the pathogens that cause HAI in this setting, pathogen detection, patient decolonization, and environmental disinfection. To further direct preventive measures, data are needed to better delineate the epidemiology of the pathogens involved in combat-injury-related infections, specifically the role of cross-contamination with these organisms within deployed MTFs and during the

transportation of the injured between facilities. Colonization screening within deployed MTFs would use valuable resources but is worth exploring. Admission and interval screening of local national patients, especially those transferred from other healthcare facilities, may be the best place to start. More rapid detection, identification, and analysis of antimicrobial susceptibility could help guide antimicrobial selection and infection prevention measures, as well as limit broad-spectrum antimicrobial use. The usefulness and effectiveness of patient cleansing/decolonization merits further study. Patient cleansing with chlorhexidine cloths is currently recommended in US military theater guidelines.[50] The impact of this intervention in decreasing MDR colonization and later infections has not been analyzed and published. The use of chlorhexidine in similar settings in civilian practice has produced mixed results;[51,52] more research is needed. Evaluation of selective oral and digestive decontamination is also an area that merits further research in this setting. Although hospital cleaning programs, with approved disinfectants, have long been establish, there are many novel technologies (e.g., vaporized hydrogen peroxide and ultraviolet light) that continue to be developed which could potentially be adopted to disinfect the sometimes unique structures of the deployed MTF. Studies on the effectiveness of most of these technologies are not readily available, and no studies of their use in the setting of the deployed MTF have been conducted.

CONCLUSIONS

Although numerous challenges are present in the deployed setting, practice of infection prevention and control should mirror that performed in hospitals outside the combat zone whenever possible. Practice should follow US and international guidelines and standards, although some modifications may be necessary based on local facility design, logistical challenges, personnel availability and skills, security, and environmental concerns.

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Executive Summary: Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update—Endorsed by the Infectious Diseases Society of America

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Abstract: Despite advances in resuscitation and surgical management of combat wounds, infection remains a concerning and potentially preventable complication of combat-related injuries. Interventions currently used to prevent these infections have not been either clearly defined or subjected to rigorous clinical trials. Current infection prevention measures and wound management practices are derived from retrospective review of wartime experiences, from civilian trauma data, and from in vitro and animal data. This update to the guidelines published in 2008 incorporates evidence that has become available since 2007. These guidelines focus on care provided within hours to days of injury, chiefly within the combat zone, to those combat-injured patients with open wounds or burns. New in this update are a consolidation of antimicrobial agent recommendations to a backbone of high-dose cefazolin with or without metronidazole for most postinjury indications and recommendations for redosing of antimicrobial agents, for use of negative pressure wound therapy, and for oxygen supplementation in flight.

Key Words: Guidelines, Infection, Combat, Trauma, Prevention.

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EXECUTIVE SUMMARY

Infectious complications of combat trauma have plagued man throughout the ages. Advances in body armor and in the medical care provided from the point of injury to definitive

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Guideline Disclaimer: It is important to realize that guidelines cannot always account for individual variation among patients. They are not intended to supplant physician judgment with respect to particular patients or special clinical situations. Adherence to these guidelines is voluntary, with the ultimate determination regarding their application to be made by the physician in the light of each patient's individual circumstances.
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care have allowed injured personnel to survive what previously would have been fatal injuries. Personnel surviving these severe injuries, which are often complex and associated with extensive tissue destruction, are at high risk for both early and remote infectious complications. Strategies to prevent these infections are chiefly derived from retrospective review of experiences in past and current conflicts, from civilian trauma data, and from in vitro and animal data. The best clinical practices to prevent infections in combat injuries have not been fully established. The following guidelines integrate available evidence and expert opinion, from the military and civilian medical community, both within and outside of the United States. These updated guidelines provide recommendations to healthcare providers for the management of combat-injured patients with open wounds or burns to prevent infectious complications. They focus on care from point of injury until arrival to tertiary care facilities outside of the combat zone. Postinjury antimicrobials, early wound cleansing (irrigation) and surgical debridement, delayed closure, and bony stabilization, with emphasis on maintenance of infection control measures,[1] are the essential components in reducing the incidence of these infections. New in this update are a consolidation of antimicrobial agent recommendations to a backbone of high-dose cefazolin with or without metronidazole for most postinjury indications and recommendations for redosing of antimicrobial agents, for use of negative pressure wound therapy (NPWT), and for oxygen supplementation in flight. Although focused on prevention of infections after injuries produced by combat, these guidelines may be applicable to noncombat traumatic injuries under certain circumstances.

Each section begins with a question and is followed by numbered recommendations from the panel with strength and quality of supporting evidence ratings (Table 1). In addition, T1
a table is included to guide use of these recommendations based on the (US military) level of medical care (Table 2). T2
Recommendations are supported by the five evidence-based reviews included in this *Journal of Trauma* supplement: (1) Prevention of infections associated with combat-related extremity injuries,[2] (2) Prevention of infections associated with combat-related central nervous system injuries,[3] (3) Prevention of infections associated with combat-related eye, maxillofacial, and neck injuries,[4] (4) Prevention of infections associated with combat-related thoracic and abdominal cavity

TABLE 1. GRADE* Systematic Weighting of the Quality of Evidence and Grading of Recommendations

Strength of Recommendation and Quality of Evidence	Methodological Quality of Supporting Evidence (Examples)	Clarity of Balance Between Desirable and Undesirable Effects
IA Strong recommendation, high-quality evidence	Consistent evidence from well-performed RCTs or exceptionally strong evidence from unbiased observational studies	Desirable effects clearly outweigh undesirable effects or vice versa
IB Strong recommendation, moderate-quality evidence	Evidence from RCTs with important limitations (inconsistent results, methodological flaws, indirect, or imprecise) or exceptionally strong evidence from unbiased observational studies	Desirable effects clearly outweigh undesirable effects or vice versa
IC Strong recommendation, low-quality evidence	Evidence for at least one critical outcome from observational studies, RCTs with serious flaws or indirect evidence	Desirable effects clearly outweigh undesirable effects or vice versa
ID Strong recommendation, very low-quality evidence	Evidence for at least one critical outcome from unsystematic clinical observations or very indirect evidence	Desirable effects clearly outweigh undesirable effects or vice versa
IIA Weak recommendation, high-quality evidence	Consistent evidence from well-performed RCTs or exceptionally strong evidence from unbiased observational studies	Desirable effects closely balanced with undesirable effects
IIB Weak recommendation, moderate-quality evidence	Evidence from RCTs with important limitations (inconsistent results, methodological flaws, indirect, or imprecise) or exceptionally strong evidence from unbiased observational studies	Desirable effects closely balanced with undesirable effects
IIC Weak recommendation, low-quality evidence	Evidence for at least one critical outcome from observational studies, from RCTs with serious flaws or indirect evidence	Uncertainty in the estimates of desirable effects, harms, and burden; desirable effects, harms, and burden may be closely balanced
IID Weak recommendation, very low-quality evidence	Evidence for at least one critical outcome from unsystematic clinical observations or very indirect evidence	Major uncertainty in the estimates of desirable effects, harms, and burden; Desirable effects may or may not be balanced with undesirable effects may be closely balanced

RCTs, randomized controlled trials.
* Grades of Recommendation, Assessment, Development, and Evaluation (GRADE), www.gradeworkinggroup.org.

TABLE 2. Recommendations to Prevent Infections Associated With Combat-Related Injuries Based on Level of Care

Level of Care*	Care Category	Recommendations
I (prehospital)	Initial care in the field	Bandage wounds with sterile dressings (avoid pressure over eye wounds) (**IB**)
		Stabilize fractures (**IB**)
		Transfer to surgical support as soon as feasible (**IB**)
	Postinjury antimicrobials	Provide single-dose point-of-injury antimicrobials (Table 3) if evacuation is delayed or expected to be delayed (**IC**)
I/II without surgical support (IIa)	Postinjury antimicrobials	Provide IV antimicrobials (Table 3) as soon as possible (within 3 h) (**IB**)
		Provide tetanus toxoid and immune globulin as appropriate (**IB**)
		Enhance Gram negative coverage with aminoglycoside or fluoroquinolone not recommended (**IB**)
		Addition of penicillin to prevent clostridial gangrene or streptococcal infection is not recommended (**IC**)
		Redose antimicrobials if large volume blood produce resuscitation (**IC**)
		Use only topical antimicrobials for burns (**IB**)
	Debridement and irrigation	Irrigate wounds to remove gross contamination with normal saline, sterile, or potable water, under low pressure (bulb syringe or equivalent) without additives (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
II with surgical support (IIb)/III	Postinjury antimicrobials	Provide IV antimicrobials (Table 3) as soon as possible (within 3 h) (**IB**)
		Provide tetanus toxoid and immune globulin as appropriate (**IB**)
		Enhance Gram negative coverage with aminoglycoside or fluoroquinolone not recommended (**IB**)
		Addition of penicillin to prevent clostridial gangrene or streptococcal infection is not recommended (**IC**)
		Redose antimicrobials if large volume blood produce resuscitation (**IC**)
		Use only topical antimicrobials for burns (**IB**)
		Antimicrobial beads or pouches may be used (**IB**)
		Provide postsplenectomy immunizations if indicated (**IB**)
	Debridement and irrigation	Irrigate wounds to remove contamination with normal saline or sterile water, under low pressure (5–10 PSI, e.g., bulb syringe or gravity flow) without additives (use 3 L for each type I, 6 L for each type II, and 9 L for each type III extremity fractures) (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
		Do not obtain cultures unless infection is suspected (**IB**)
	Surgical wound management	Surgical evaluation as soon as possible (**IB**)
		Only dural and facial wounds should undergo primary closure (**IB**)
		NPWT can be used (**IB**)
		External fixation (temporary spanning) of femur/tibia fractures (**IB**)
		External fixation (temporary spanning) or splint immobilization of open humerus/forearm fractures (**IB**)
IV	Postinjury antimicrobials	Complete course of postinjury antimicrobials (Table 3)
		Antimicrobial beads or pouches may be used (**IB**)
		Provide postsplenectomy immunizations if indicated (**IB**)
	Debridement and irrigation	Irrigate wounds to remove contamination with normal saline or sterile water, under low pressure (5–10 PSI, e.g., bulb syringe or gravity flow) without additives (use 3 L for each type I, 6 L for each type II, and 9 L for each type III extremity fractures) (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
		Do not obtain cultures unless infection is suspected (**IB**)
	Surgical wound management	Wounds should not be closed until 3–5 d postinjury (**IB**)
		Only dural and facial wounds should undergo primary closure (**IB**)
		NPWT can be used (**IB**)
		External fixation (temporary spanning) of femur/tibia fractures (**IB**)
		External fixation (temporary spanning) or splint immobilization of open humerus/forearm fractures (**IB**)

IV, intravenous; PSI, pounds per square inch.

* Role of care, level of care, and echelon of care are considered synonymous with role currently the preferred US military term. Definitions of role/level/echelon of care: *Role 1*—self-aid, buddy aid, combat lifesaver, and combat medic/corpsman care at the point-of-injury; physician/physician assistant care at battalion aid station (BAS; US Army) or shock trauma platoon (US Marine Corps [USMC]); no patient holding capacity; *Role 2*—medical company (includes forward support medical company, main support medical company, and area support medical company in US Army) or expeditionary medical support (EMEDS, US Air Force [USAF]); 72 h patient holding capacity, basic blood transfusion, radiography, and laboratory support. May be supplemented with surgical assets (2b) (forward surgical team, US Army; mobile field surgical team, USAF; forward resuscitative surgical system, USMC); *Role 3*—combat support hospital (CSH, US Army), Air Force theater hospital (AFTH, USAF), or casualty receiving ships (USN); full inpatient capacity with intensive care units and operating rooms; *Role 4*—regional hospital (Landstuhl Regional Medical Center, Germany) or USNS hospital ships (USN), typically outside of the combat zone; general and specialized inpatient medical and surgical care; *Role 5*—care facilities within United States, typically tertiary care medical centers.

† Criteria for allowing retained fragments to remain behind: entry/exit wounds <2 cm; no bone, joint, vascular, and body cavity involvement; no high-risk etiology (e.g., mine); no obvious infection; and assessable by X-ray.

injuries,[5] and (5) Prevention of infections associated with combat-related burn injuries.[6]

RECOMMENDATIONS FOR THE PREVENTION OF INFECTIONS ASSOCIATED WITH COMBAT-RELATED INJURIES

A. Initial Care in the Field

I. What Initial Care/Stabilization Should be Provided to the Injured Patient in the Field Before Evacuation to a Medical Care Facility (Medical Treatment Facilities)?

1. Wounds should be bandaged with sterile dressing and fractures stabilized before transportation to higher level of care (**IB**) (Table 2).
2. Dressing covering the eye should provide protection while avoiding producing pressure on the orbit (**IB**). A Fox shield or other such device should be employed.
3. Patients should be transferred to a facility with surgical support as soon as feasible (**IB**) (see recommendation 44).
4. Given the unpredictable nature of casualty evacuation in a combat zone, point-of-injury antimicrobial agents (see recommendation 20) should be provided if evacuation is delayed or expected to be delayed (**IC**).

B. Postinjury Antimicrobials

II. Should Systemic Antimicrobials be Given to Patients With Combat-Related Injuries Immediately Postinjury?

5. Systemic antimicrobials should be administered as soon as possible after injury to prevent early infectious complications, including sepsis, caused by common bacterial flora. Ideally, postinjury antimicrobials should be given within 3 hours of injury (**IB**).

III. Which Antimicrobials (and What Dosing Regimens) Should be Employed for Postinjury Use?

6. Antimicrobial selection should focus on providing the narrowest spectrum of activity required, providing coverage of expected common bacterial flora. If multiple injuries are present, the antimicrobial agent selection should be based on the narrowest spectrum needed to cover all wound sites/types (**IB**). Postinjury antimicrobials are provided to prevent early infectious complications, including sepsis. These recommended antimicrobials are not meant to treat established infections where nosocomial pathogens, including multidrug-resistant, may be the infecting agents (Table 3). T3
7. Selected agents should be dosed to maximize pharmacokinetics and pharmacodynamics. Logistical considerations, including limiting number of agents to be stocked and maintaining sufficient quantities in the combat zone, should also be considered.

Extremity Wounds

8. Cefazolin, 2 g intravenously (IV) every 6 hours to 8 hours, should be used as the antimicrobial of choice in extremity injuries (skin, soft tissue, and/or bone) (**IB**). Clindamycin may be given as an alternate agent if previous documented anaphylaxis to β-lactam antimicrobials.
9. Enhanced gram-negative coverage should not be employed (**IB**).
10. Addition of penicillin to provide antimicrobial coverage of clostridial gangrene and group A β-hemolytic *Streptococcus* infections is not required (**IC**).

Central Nervous System Wounds

11. Cefazolin, 2 g IV every 6 hours to 8 hours, should be employed for central nervous system (CNS) injuries (**IB**).
12. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if brain grossly contaminated with organic debris (**ID**).
13. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if spinal cord injury associated with concomitant abdominal cavity penetration (**IC**).

Eye, Maxillofacial, and Neck Wounds

14. For penetrating eye injuries, levofloxacin, 500 mg IV or orally every 24 hours, should be provided (**IB**).
15. For maxillofacial and neck injuries, cefazolin, 2 g IV every 6 hours to 8 hours, should be provided (**IC**). Clindamycin, 600 mg IV every 8 hours, may be used as an alternate (**IC**).

Thoracic and Abdominal Cavity Wounds

16. For thoracic cavity injuries without disruption of the esophagus, cefazolin, 2 g IV every 6 hours to 8 hours, should be used (**IIB**).
17. Cefazolin, 2 g IV every 6 hours to 8 hours, with metronidazole, 500 mg IV every 8 hours to 12 hours, should be provided for penetrating wounds to the abdomen and penetrating wounds to the thorax that result in esophageal injury (**IIB**). Alternate regimens include single-dose ertapenem (1 g IV) or moxifloxacin (400 mg IV) (**IIB**).

Burns

18. Topical antimicrobial agents should be used for burn wounds in conjunction with debridement (**IB**). Silver sulfadiazine cream alternating with mafenide acetate cream is preferred. Debridement may not be feasible at lower levels of care; in this situation, clean, dry dressing should be applied to burn wound until the patient is transferred to a higher level of care.
19. Systemic antimicrobials are not indicated for postinjury therapy (**IC**), or for debridement performed as part of routine wound care (**IB**), unless required for concomitant traumatic injuries. Systemic antimicrobials may be considered for perioperative prophylaxis during excision and grafting procedures (**IC**). Cefazolin, 2 g IV every 6 hours to 8 hours for 24 hours, is sufficient for coverage of skin flora. However, antimicrobial agents effective against *Pseudomonas* should be considered if wounds are grossly colonized or older than 5 days. AQ: 4

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TABLE 3. Postinjury Antimicrobial Agent Selection and Duration Based Upon Injury Pattern*

Injury	Preferred Agent(s)	Alternate Agent(s)	Duration
Extremity wounds (includes skin, soft tissue, and bone)			
Skin, soft tissue, no open fractures	Cefazolin, 2 g IV q 6–8 h†‡	Clindamycin (300–450 mg PO TID or 600 mg IV q 8 h)	1–3 d
Skin, soft tissue, with open fractures, exposed bone, or open joints	Cefazolin 2 g IV q 6–8 h†‡§	Clindamycin 600 mg IV q 8 h	1–3 d
Thoracic wounds			
Penetrating chest injury without esophageal disruption	Cefazolin, 2 g IV q 6–8 h†‡	Clindamycin (300–450 mg PO TID or 600 mg IV q 8 h)	1 d
Penetrating chest injury with esophageal disruption	Cefazolin 2 g IV q 6–8 h†‡ plus metronidazole 500 mg IV q 8–12 h	Ertapenem 1 g IV × 1 dose or moxifloxacin 400 mg IV × 1 dose	1 d after definitive washout
Abdominal wounds			
Penetrating abdominal injury with suspected/known hollow viscus injury and soilage; may apply to rectal/perineal injuries as well	Cefazolin 2 g IV q 6–8 h†‡ plus metronidazole 500 mg IV q 8–12 h	Ertapenem 1 g IV × 1 dose or moxifloxacin 400 mg IV × 1 dose	1 d after definitive washout
Maxillofacial and neck wounds			
Open maxillofacial fractures, or maxillofacial fractures with foreign body or fixation device	Cefazolin 2 g IV q 6–8 h†‡	Clindamycin 600 mg IV q 8 h	1 d
Central nervous system wounds			
Penetrating brain injury	Cefazolin 2 g IV q 6–8 h.†‡ Consider adding metronidazole 500 mg IV q 8–12 h if gross contamination with organic debris	Ceftriaxone 2 g IV q 24 h. Consider adding metronidazole 500 mg IV q 8–12 h if gross contamination with organic debris. For penicillin allergic patients, vancomycin 1 g IV q 12 h plus ciprofloxacin 400 mg IV q 8–12 h	5 d or until CSF leak is closed, whichever is longer
Penetrating spinal cord injury	Cefazolin 2 g IV q 6–8 h.†‡ ADD metronidazole 500 mg IV q 8–12 h if abdominal cavity is involved	As above. ADD metronidazole 500 mg IV q 8–12 h if abdominal cavity is involved	5 d or until CSF leak is closed, whichever is longer
Eye wounds			
Eye injury, burn or abrasion	Topical: Erythromycin or Bacitracin ophthalmic ointment QID and PRN for symptomatic relief Systemic: No systemic treatment required	Fluoroquinolone 1 drop QID	Until epithelium healed (no fluorescein staining)
Eye injury, penetrating	Levofloxacin 500 mg IV/PO once daily. Before primary repair, no topical agents should be used unless directed by ophthalmology		7 d or until evaluated by a retinal specialist
Burns			
Superficial burns	Topical antimicrobials with twice daily dressing changes (include mafenide acetate‖ or silver sulfadiazine; may alternate between the two), silver-impregnated dressing changed q 3–5d, or Biobrane	Silver nitrate solution applied to dressings	Until healed
Deep partial-thickness burns	Topical antimicrobials with twice daily dressing changes, or silver-impregnated dressing changed q 3–5d, plus excision and grafting	Silver nitrate solution applied to dressings plus excision and grafting	Until healed or grafted
Full-thickness burns	Topical antimicrobials with twice daily dressing changes plus excision and grafting	Silver nitrate solution applied to dressings plus excision and grafting	Until healed or grafted

TABLE 3. Postinjury Antimicrobial Agent Selection and Duration Based Upon Injury Pattern* (continued)

Injury	Preferred Agent(s)	Alternate Agent(s)	Duration
Point-of-injury/delayed evacuation¶			
Expected delay to reach surgical care	Moxifloxacin 400 mg PO × 1 dose. Ertapenem 1 g IV or IM if penetrating abdominal injury, shock, or unable to tolerate PO medications	Levofloxacin 500 mg PO × 1 dose. Cefotetan 2 g IV or IM q 12 h if penetrating abdominal injury, shock, or unable to tolerate PO medications	Single-dose therapy

IV, intravenous; PO, orally; IM, intramuscularly; TID, three times daily; QID, four times daily; PRN, as needed; CSF, cerebrospinal fluid.

* Postinjury antimicrobial agents are recommended to prevent early posttraumatic infectious complications, including sepsis, secondary to common bacterial flora. Selection is based on narrowest spectrum and duration required to prevent early infections before adequate surgical wound management. This narrow spectrum is selected to avoid selection of resistant bacteria. The antimicrobials listed are not intended for use in established infections, where multidrug-resistant or other nosocomial pathogens may be causing infection.

† Cefazolin may be dosed based on body mass: 1 g if weight ≤80 kg (176 lbs), 2 g if weight 81–160 kg (177–352 lbs), 3 g if weight >160 kg (>352 lbs); doses up to 12 g daily are supported by FDA-approved package insert.

‡ Pediatric dosing: cefazolin, 20–30 mg/kg IV q 6–8 h (maximum, 100 mg/kg/d); metronidazole, 7.5 mg/kg IV q 6 h; clindamycin 25–40 mg/kg/d IV divided q 6–8 h; ertapenem, 15 mg/kg IV or IM q 12 (children up to 12 yr) or 20 mg/kg IV or IM once daily (children over 12 yr; maximum, 1 g/d); ceftriaxone, 100 mg/kg/d IV divided q 12–24 h (dosing for CNS injury); levofloxacin, 8 mg/kg IV or PO q 12 h (levofloxacin is only FDA-approved in children for prophylaxis of inhalational anthrax in children older than 6 mo, but this dose is commonly used for other indications); vancomycin, 60 mg/kg/d IV divided q 6 h (dosing for CNS injury); ciprofloxacin, 10 mg/kg IV (or 10–20 mg/kg PO) q 12 h.

§ These guidelines do not advocate adding enhanced Gram negative bacteria coverage (i.e., addition of fluoroquinolone or aminoglycoside antimicrobials) in type III fractures.

‖ Mafenide acetate is contraindicated in infants younger than 2 mo.

¶ Postinjury antimicrobial therapy as suggested by the Tactical Combat Casualty Care Committee.

Point-of-Injury Antimicrobial Selection

20. Point-of-injury antimicrobials as suggested by the Tactical Combat Casualty Care Committee currently include moxifloxacin, 400 mg orally, if casualty does not have penetrating abdominal trauma, is not in shock, and can take oral medications. In patients who do not meet these criteria, single-dose ertapenem (1 g IV or intramuscularly [IM]) or cefotetan (2 g IV or IM) every 12 hours has been suggested. IV therapy is preferred over IM.

Pediatric Considerations

21. Children should be treated with the same antimicrobial agents as those suggested for adults, including those topical antimicrobials suggested for burns. Dosing of antimicrobials in children weighing less than 40 kg should be weight-based. Cefazolin should be dosed at 20 mg/kg to 30 mg/kg IV every 6 hours to 8 hours (up to maximum of 100 mg/kg/d). Metronidazole should be dosed at 30 mg/kg/d IV in four divided doses.

IV. What Duration of Antimicrobials Should be Given to Patients After Combat-Related Injuries?

22. The shortest course of postinjury antimicrobial therapy should be used (**IB**) (Table 3). If multiple wounds are present, the duration of antimicrobials is dictated by the injury pattern requiring the longest duration of therapy. Duration should not be extended for open wounds, drains, or external fixation devices. Wounds should be continually reassessed for evidence of infection and antimicrobials directed specifically at known or empirically suspected infecting pathogens provided if infection is suspected or proven.

Extremity Wounds

23. Antimicrobials should be provided for 1 day to 3 days for all extremity wounds (**IB**).

CNS Wounds

24. Antimicrobials are recommended for 5 days or until cerebrospinal fluid leak is closed, whichever time period is longer (**ID**).

Eye, Maxillofacial, and Neck Wounds

25. For penetrating eye injuries, antimicrobials should be provided for a total of 7 days or until a thorough evaluation by a retinal specialist with adequate capabilities has been performed (**IC**).
26. For maxillofacial and neck injuries, 1 day of antimicrobial coverage should be provided (**IC**).

Thoracic and Abdominal Cavity Wounds

27. Thoracic injuries with esophageal injury should also receive a total of 1 day of antimicrobials after definitive operative washout (**IB**).
28. Casualties should receive a total of 1 day of antimicrobials after definitive operative washout for abdominal cavity injuries (**IB**).

Burns

29. Topical antimicrobial agents should be used for burns until wounds are successfully covered with healed skin, whether spontaneously or following successful skin grafting (**IC**).

V. Should Antimicrobials be Redosed Before Next Schedule Dosing Interval if Patients Require Substantial Blood Product Support, Require Large Volume Resuscitation, or Have Severe Acidosis?

30. Redosing of antimicrobials should be performed after large volume blood product resuscitation (1,500–2,000 mL of blood loss) has been completed, regardless of when the last dose of antimicrobial was administered (**IC**).

VI. Should Local Delivery of Antimicrobials Through Topical Application or Beads (Bead Pouches) be Implemented in the Care of Combat-Related Injuries?

31. Local delivery of topical antimicrobials may be provided for extremity infections in the form of antimicrobial beads or pouches as long as the emphasis is still on surgical debridement and irrigation (**IB**).
32. Local delivery of other antimicrobials (other than in burn care), to include powders or soaking of wet to dry dressing with antimicrobials, should not be used routinely (**IB**).

VII. What Vaccines or Other Immunotherapy Should be Provided Postinjury?

Tetanus Toxoid or Immune Globulin

33. Patients who have been previously immunized against tetanus (received 3 or more doses of toxoid) do not require booster dose of vaccine unless it has been more than 5 years since their last dose. They do not require tetanus immune globulin (TIG) (**IB**).
34. Unimmunized patients, and those with unknown vaccination status, should receive TIG and vaccine (with additional doses of vaccine given at 4 weeks and 6 months) postinjury (**IC**).
35. Early surgical debridement and irrigation in addition to postinjury antimicrobials and vaccine may be effective in the prevention of tetanus in the absence of TIG administration (**IID**).

Postsplenectomy Immunization

36. Patients who have had their spleens removed should receive immunization against *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Hemophilus influenza* serotype B (**IB**). Immunization should be provided within 14 days of splenectomy.

C. Debridement and Irrigation

VIII. When Should Irrigation Fluid be Implemented in the Management of Combat-Related Injuries?

37. Wound irrigation should be initiated as soon as clinically possible by appropriately trained personnel (**ID**).

IX. Should Additives Supplement Irrigation Fluid for Combat-Related Injuries?

38. Additives should not be included in standard irrigation fluid as normal saline (or alternately, sterile water or potable water) is adequate (**IB**).

X. What Volume of Fluid Should be Used to Irrigate Wounds Associated With Combat Injuries?

39. Sufficient volume to remove debris should be employed (**IB**). For extremity injuries, standard volumes of 3 L, 6 L, and 9 L should be provided for Type I, II, and III fractures, respectively; however, larger volumes might be required for more severe injuries (**IB**).

XI. What Pressure Should be Used to Deliver Irrigation in the Management of Combat-Related Injuries?

40. Irrigation fluid should be delivered at low pressure (5–10 PSI may be delivered by bulb syringe or gravity irrigation) (**IB**).

XII. Should Pre- and/or Postdebridement Bacterial Culture of Combat-Related Wounds be Performed?

41. Clinicians should obtain bacterial cultures only when there are concerns for an ongoing wound infection based upon systemic signs or symptoms of infection, local appearance of wounds, and laboratory or radiographic imaging studies (**IB**).
42. Results from infection control surveillance cultures should not be used for initiation of therapy (**IC**).

XIII. Can Retained Soft Tissue Fragments Remain in a Combat-Related Injury Wound?

43. Casualties with isolated retained deep extremity soft tissue metal fragments meeting certain clinical and radiographic criteria should be treated with a single dose of cefazolin, 2 g IV, without fragment removal (**IB**). Patients should be monitored for evidence of subsequent infection.

D. Surgical Wound Management

XIV. When Should Patients With Combat-Related Injuries Undergo Initial Surgical Management?

44. Patients should be evacuated to surgical care as soon as possible based upon a risk-benefit analysis of the combat environment (**IB**).

45. Penetrating injuries of the eye (**IB**) and spine without neurologic compromise (**IC**) should await surgical debridement until appropriate surgical expertise is available.
46. Foreign material embedded in the brain, which are not readily accessible, should not be removed by non-neurosurgeons (**IB**).
47. All burn injuries should undergo thorough cleansing and debridement, estimation of extent and depth, and coverage with appropriate topical antimicrobial agents within 8 hours of injury (**IC**). Early (within 5 days) excision and grafting is suggested for deep partial-thickness and full-thickness burns (**IA**). This should ideally be performed outside of the combat zone by surgeons with appropriate training and experience.

XV. When Should Combat-Related Wounds be Closed?

48. Wounds, to include open fractures, should not be closed early; typical closure should be performed 3 days to 5 days after injury if there is no evidence of infection (**IB**).
49. For injuries that involve the face or dura, primary closure should be performed (**IB**).
50. For abdominal and thoracic injuries, the skin should not be closed if there is a colon injury or extensive devitalized tissue due to excessive infectious complications (**IB**).
51. Early primary repair of complex or destructive colonic injuries should not be performed especially if associated with massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high velocity injury, or extensive local tissue damage (**IB**).
52. If the abdomen is left open, the possibility of partial or complete closure should be considered at each subsequent laparotomy (**IB**).
53. Scheduled laparotomies should be performed in this group at 24-hour to 48-hour intervals (**IB**).

XVI. Should External Fixation be Standard for Stabilization of Fracture?

54. Temporary spanning external fixation should be placed for femoral and tibial fractures (**IB**). Use of external fixation in the current conflicts allows stabilization during long evacuations to the United States, easy observation of wounds (over use of plaster), and potentially less chronic infections (over early open reduction and internal fixation).
55. Temporary spanning external fixation or splint immobilization placement with transition to open plate and screw osteosynthesis should be employed for open humerus and forearm fractures after soft tissue stabilization (**IB**).

XVII. Can NPWT be Used in the Management of Combat-Related Wounds?

56. NPWT should be used in the management of open wounds (excluding CNS injuries) to include during aeromedical evacuation of patients (**IB**).
57. Use of intermittent suction or instillation of normal saline in conjunction with NPWT is discouraged in most situations based upon preliminary animal studies (**ID**).
58. Local delivery of antimicrobials using beads or pouches might be effective in combination with NPWT and could be considered (**IID**).

XVIII. Should Supplemental Oxygen be Provided During Transportation of the Wounded to Medical Facilities Outside the Combat Zone?

59. During aeromedical evacuation, supplemental oxygen (to maintain oxygen saturation >92%) may be beneficial in patients with combat-related injuries (**IIC**).

E. Facility Infection Control and Prevention

XIX. What Infection Control and Prevention Measures Should be Implemented in Deployed Medical Treatment Facilities?

60. Basic infection control and prevention measures should be employed at all deployed medical treatment facilities (MTF). These should include hand hygiene, with compliance monitoring. Infection control and prevention should include MTF Commander oversight and emphasis (**IB**).
61. Transmission-based (isolation) precautions should be implemented (**IB**).
62. Cohorting (i.e., physically separating patients expected to be hospitalized for less than 72 hours from those expected to be hospitalized longer) should be used (**IC**).
63. An infection control officer should be assigned to each deployed MTF that provides inpatient care. This officer should have adequate training and experience to lead the infection control program at the MTF.
64. All deployed MTF should practice antimicrobial stewardship (**IC**). Clinical microbiology assets are crucial to antimicrobial stewardship and should be available at MTF which hospitalize patients for more than 72 hours.

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Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update—Endorsed by the Infectious Diseases Society of America

AQ: 1

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Abstract: Despite advances in resuscitation and surgical management of combat wounds, infection remains a concerning and potentially preventable complication of combat-related injuries. Interventions currently used to prevent these infections have not been either clearly defined or subjected to rigorous clinical trials. Current infection prevention measures and wound management practices are derived from retrospective review of wartime experiences, from civilian trauma data, and from in vitro and animal data. This update to the guidelines published in 2008 incorporates evidence that has become available since 2007. These guidelines focus on care provided within hours to days of injury, chiefly within the combat zone, to those combat-injured patients with open wounds or burns. New in this update are a consolidation of antimicrobial agent recommendations to a backbone of high-dose cefazolin with or without metronidazole for most postinjury indications, and recommendations for redosing of antimicrobial agents, for use of negative pressure wound therapy, and for oxygen supplementation in flight.

Key Words: Guidelines, Infection, Combat, Trauma, Prevention.

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EXECUTIVE SUMMARY

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Infectious complications of combat trauma have plagued man throughout the ages. Advances in body armor and in the medical care provided from the point-of-injury to definitive care have allowed injured personnel to survive what previously would have been fatal injuries. Personnel surviving these severe injuries, which are often complex and associated with extensive tissue destruction, are at high risk for both early and remote infectious complications. Strategies to prevent these infections are chiefly derived from retrospec-

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TABLE 1. GRADE* Systematic Weighting of the Quality of Evidence and Grading of Recommendations

Strength of Recommendation and Quality of Evidence		Methodological Quality of Supporting Evidence (Examples)	Clarity of Balance Between Desirable and Undesirable Effects
IA	Strong recommendation, high-quality evidence	Consistent evidence from well-performed RCTs or exceptionally strong evidence from unbiased observational studies	Desirable effects clearly outweigh undesirable effects or vice versa
IB	Strong recommendation, moderate-quality evidence	Evidence from RCTs with important limitations (inconsistent results, methodological flaws, indirect, or imprecise) or exceptionally strong evidence from unbiased observational studies	Desirable effects clearly outweigh undesirable effects or vice versa
IC	Strong recommendation, low-quality evidence	Evidence for at least one critical outcome from observational studies, RCTs with serious flaws or indirect evidence	Desirable effects clearly outweigh undesirable effects or vice versa
ID	Strong recommendation, very low-quality evidence	Evidence for at least one critical outcome from unsystematic clinical observations or very indirect evidence	Desirable effects clearly outweigh undesirable effects or vice versa
IIA	Weak recommendation, high-quality evidence	Consistent evidence from well-performed RCTs or exceptionally strong evidence from unbiased observational studies	Desirable effects closely balanced with undesirable effects
IIB	Weak recommendation, moderate-quality evidence	Evidence from RCTs with important limitations (inconsistent results, methodological flaws, indirect, or imprecise) or exceptionally strong evidence from unbiased observational studies	Desirable effects closely balanced with undesirable effects
IIC	Weak recommendation, low-quality evidence	Evidence for at least one critical outcome from observational studies, from RCTs with serious flaws or indirect evidence	Uncertainty in the estimates of desirable effects, harms, and burden; desirable effects, harms, and burden may be closely balanced
IID	Weak recommendation, very low-quality evidence	Evidence for at least one critical outcome from unsystematic clinical observations or very indirect evidence	Major uncertainty in the estimates of desirable effects, harms, and burden; Desirable effects may or may not be balanced with undesirable effects may be closely balanced

RCTs, randomized controlled trials.
* Grades of Recommendation, Assessment, Development, and Evaluation (GRADE), www.gradeworkinggroup.org.

tive review of experiences in past and current conflicts, from civilian trauma data, and from in vitro and animal data. The best clinical practices to prevent infections in combat injuries have not been fully established. The following guidelines integrate available evidence and expert opinion, from the military and civilian medical community, both within and outside of the United States. These updated guidelines provide recommendations to healthcare providers for the management of combat-injured patients with open wounds or burns to prevent infectious complications. They focus on care from point-of-injury until arrival to tertiary care facilities outside of the combat zone. Postinjury antimicrobials, early wound cleansing (irrigation) and surgical debridement, delayed closure, and bony stabilization, with emphasis on maintenance of infection control measures,[1] are the essential components in reducing the incidence of these infections. New in this update are a consolidation of antimicrobial agent recommendations to a backbone of high-dose cefazolin with or without metronidazole for most postinjury indications and recommendations for redosing of antimicrobial agents, for use of negative pressure wound therapy (NPWT), and for oxygen supplementation in flight. Although focused on prevention of infections after injuries produced by combat, these guidelines may be applicable to noncombat traumatic injuries under certain circumstances.

Each section begins with a question and is followed by numbered recommendations from the panel with strength and quality of supporting evidence ratings (Table 1). In addition, a table is included to guide use of these recommendations based on the (US military) level of medical care (Table 2). Recommendations are supported by the five evidence-based reviews included in this *Journal of Trauma* supplement: (1) Prevention of infections associated with combat-related extremity injuries,[2] (2) Prevention of infections associated with combat-related central nervous system injuries,[3] (3) Prevention of infections associated with combat-related eye, maxillofacial, and neck injuries,[4] (4) Prevention of infections associated with combat-related thoracic and abdominal cavity injuries,[5] and (5) Prevention of infections associated with combat-related burn injuries.[6]

RECOMMENDATIONS FOR THE PREVENTION OF INFECTIONS ASSOCIATED WITH COMBAT-RELATED INJURIES

A. Initial Care in the Field

I. What Initial Care/Stabilization Should be Provided to the Injured Patient in the Field Before Evacuation to a Medical Care Facility (Medical Treatment Facilities)?

1. Wounds should be bandaged with sterile dressing and fractures stabilized before transportation to higher level of care (**IB**) (Table 2).
2. Dressing covering the eye should provide protection while avoiding producing pressure on the orbit (**IB**). A Fox shield or other such device should be employed.

TABLE 2. Recommendations to Prevent Infections Associated With Combat-Related Injuries Based on Level of Care

Level of Care*	Care Category	Recommendations
I (prehospital)	Initial care in the field	-Bandage wounds with sterile dressings (avoid pressure over eye wounds) (**IB**)
		Stabilize fractures (**IB**)
		Transfer to surgical support as soon as feasible (**IB**)
	Postinjury antimicrobials	Provide single-dose point-of-injury antimicrobials (Table 3) if evacuation is delayed or expected to be delayed (**IC**)
I/II without surgical support (IIa)	Postinjury antimicrobials	Provide IV antimicrobials (Table 3) as soon as possible (within 3 h) (**IB**)
		Provide tetanus toxoid and immune globulin as appropriate (**IB**)
		Enhance gram-negative coverage with aminoglycoside or fluoroquinolone not recommended (**IB**)
		Addition of penicillin to prevent clostridial gangrene or streptococcal infection is not recommended (**IC**)
		Redose antimicrobials if large volume blood produce resuscitation (**IC**)
		Use only topical antimicrobials for burns (**IB**)
	Debridement and irrigation	Irrigate wounds to remove gross contamination with normal saline, sterile, or potable water, under low pressure (bulb syringe or equivalent) without additives (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
II with surgical support (IIb)/III	Postinjury antimicrobials	Provide IV antimicrobials (Table 3) as soon as possible (within 3 h) (**IB**)
		Provide tetanus toxoid and immune globulin as appropriate (**IB**)
		Enhance gram-negative coverage with aminoglycoside or fluoroquinolone not recommended (**IB**)
		Addition of penicillin to prevent clostridial gangrene or streptococcal infection is not recommended (**IC**)
		Redose antimicrobials if large volume blood produce resuscitation (**IC**)
		Use only topical antimicrobials for burns (**IB**)
		Antimicrobial beads or pouches may be used (**IB**)
		Provide postsplenectomy immunizations if indicated (**IB**)
	Debridement and irrigation	Irrigate wounds to remove contamination with normal saline or sterile water, under low pressure (5–10 PSI, e.g., bulb syringe or gravity flow) without additives (use 3 L for each type I, 6 L for each type II, and 9 L for each type III extremity fractures) (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
		Do not obtain cultures unless infection is suspected (**IB**)
	Surgical wound management	Surgical evaluation as soon as possible (**IB**)
		Only dural and facial wounds should undergo primary closure (**IB**)
		NPWT can be used (**IB**)
		External fixation (temporary spanning) of femur/tibia fractures (**IB**)
		External fixation (temporary spanning) or splint immobilization of open humerus/forearm fractures (**IB**)
IV	Postinjury antimicrobials	Complete course of postinjury antimicrobials (Table 3)
		Antimicrobial beads or pouches may be used (**IB**)
		Provide postsplenectomy immunizations if indicated (**IB**)
	Debridement and irrigation	Irrigate wounds to remove contamination with normal saline or sterile water, under low pressure (5–10 PSI, e.g., bulb syringe or gravity flow) without additives (use 3 L for each type I, 6 L for each type II, and 9 L for each type III extremity fractures) (**IB**)
		Do not attempt to remove retained deep soft tissue fragments if criteria met (**IB**).† Provide cefazolin 2 g IV × 1 dose
		Do not obtain cultures unless infection is suspected (**IB**)
	Surgical wound management	Wounds should not be closed until 3–5 d postinjury (**IB**)
		Only dural and facial wounds should undergo primary closure (**IB**)
		NPWT can be used (**IB**)
		External fixation (temporary spanning) of femur/tibia fractures (**IB**)
		External fixation (temporary spanning) or splint immobilization of open humerus/forearm fractures (**IB**)

IV, intravenous; PSI, pounds per square inch.

* Role of care, level of care, and echelon of care are considered synonymous with role currently the preferred US military term. Definitions of role/level/echelon of care: *Role 1*—self-aid, buddy aid, combat lifesaver, and combat medic/corpsman care at the point-of-injury; physician/physician assistant care at battalion aid station (BAS; US Army) or shock trauma platoon (US Marine Corps [USMC]); no patient holding capacity; *Role 2*—medical company (includes forward support medical company, main support medical company, and area support medical company in US Army) or expeditionary medical support (EMEDS, US Air Force [USAF]); 72 h patient holding capacity, basic blood transfusion, radiography, and laboratory support. May be supplemented with surgical assets (2b) (forward surgical team, US Army; mobile field surgical team, USAF; forward resuscitative surgical system, USMC); *Role 3*—combat support hospital (CSH, US Army), Air Force theater hospital (AFTH, USAF), or casualty receiving ships (USN); full inpatient capacity with intensive care units and operating rooms; *Role 4*—regional hospital (Landstuhl Regional Medical Center, Germany) or USNS hospital ships (USN), typically outside of the combat zone; general and specialized inpatient medical and surgical care; *Role 5*—care facilities within United States, typically tertiary care medical centers.

† Criteria for allowing retained fragments to remain behind: entry/exit wounds < 2 cm; no bone, joint, vascular, and body cavity involvement; no high-risk etiology (e.g., mine); no obvious infection; and assessable by X-ray.

3. Patients should be transferred to a facility with surgical support as soon as feasible (**IB**) (see recommendation 44).
4. Given the unpredictable nature of casualty evacuation in a combat zone, point-of-injury antimicrobial agents (see recommendation 20) should be provided if evacuation is delayed or expected to be delayed (**IC**).

B. Postinjury Antimicrobials

II. Should Systemic Antimicrobials be Given to Patients With Combat-Related Injuries Immediately Postinjury?

5. Systemic antimicrobials should be administered as soon as possible after injury to prevent early infectious complications, including sepsis, caused by common bacterial flora. Ideally, postinjury antimicrobials should be given within 3 hours of injury (**IB**).

III. Which Antimicrobials (and What Dosing Regimens) Should be Employed for Postinjury Use?

6. Antimicrobial selection should focus on providing the narrowest spectrum of activity required, providing coverage of expected common bacterial flora. If multiple injuries are present, the antimicrobial agent selection should be based on the narrowest spectrum needed to cover all wound sites/types (**IB**). Postinjury antimicrobials are provided to prevent early infectious complications, including sepsis. These recommended antimicrobials are not meant to treat established infections where nosocomial pathogens, including multidrug-resistant (MDR), may be the infecting agents (Table 3). T3
7. Selected agents should be dosed to maximize pharmacokinetics and pharmacodynamics. Logistical considerations, including limiting number of agents to be stocked and maintaining sufficient quantities in the combat zone, should also be considered.

Extremity Wounds

8. Cefazolin, 2 g intravenously (IV) every 6 hours to 8 hours, should be used as the antimicrobial of choice in extremity injuries (skin, soft tissue, and/or bone) (**IB**). Clindamycin may be given as an alternate agent if previous documented anaphylaxis to β-lactam antimicrobials.
9. Enhanced gram-negative coverage should not be employed (**IB**).
10. Addition of penicillin to provide antimicrobial coverage of clostridial gangrene and group A β-hemolytic *Streptococcus* infections is not required (**IC**).

Central Nervous System Wounds

11. Cefazolin, 2 g IV every 6 hours to 8 hours, should be employed for central nervous system (CNS) injuries (**IB**).
12. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if brain grossly contaminated with organic debris (**ID**).
13. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if spinal cord injury associated with concomitant abdominal cavity penetration (**IC**).

Eye, Maxillofacial, and Neck Wounds

14. For penetrating eye injuries, levofloxacin, 500 mg IV or orally every 24 hours, should be provided (**IB**).
15. For maxillofacial and neck injuries, cefazolin, 2 g IV every 6 hours to 8 hours, should be provided (**IC**). Clindamycin, 600 mg IV every 8 hours, may be used as an alternate (**IC**).

Thoracic and Abdominal Cavity Wounds

16. For thoracic cavity injuries without disruption of the esophagus, cefazolin, 2 g IV every 6 hours to 8 hours, should be used (**IIB**).
17. Cefazolin, 2 g IV every 6 hours to 8 hours, with metronidazole, 500 mg IV every 8 hours to 12 hours, should be provided for penetrating wounds to the abdomen and penetrating wounds to the thorax that result in esophageal injury (**IIB**). Alternate regimens include single-dose ertapenem (1 g IV) or moxifloxacin (400 mg IV) (**IIB**).

Burns

18. Topical antimicrobial agents should be used for burn wounds in conjunction with debridement (**IB**). Silver sulfadiazine cream alternating with mafenide acetate cream is preferred. Debridement may not be feasible at lower levels of care; in this situation, clean, dry dressing should be applied to burn wound until the patient is transferred to a higher level of care.
19. Systemic antimicrobials are not indicated for postinjury therapy (**IC**), or for debridement performed as part of routine wound care (**IB**), unless required for concomitant traumatic injuries. Systemic antimicrobials may be considered for perioperative prophylaxis during excision and grafting procedures (**IC**). Cefazolin, 2 g IV every 6 hours to 8 hours for 24 hours, is sufficient for coverage of skin flora. However, antimicrobial agents effective against *Pseudomonas* should be considered if wounds are grossly colonized or older than 5 days. AQ: 3

Point-of-Injury Antimicrobial Selection

20. Point-of-injury antimicrobials as suggested by the Tactical Combat Casualty Care (TCCC) Committee currently include moxifloxacin, 400 mg orally, if casualty does not have penetrating abdominal trauma, is not in shock, and can take oral medications. In patients who do not meet these criteria, single-dose ertapenem (1 g IV or intramuscularly [IM]) or cefotetan (2 g IV or IM) every 12 hours has been suggested. IV therapy is preferred over IM.

Pediatric Considerations

21. Children should be treated with the same antimicrobial agents as those suggested for adults, including those topical antimicrobials suggested for burns. Dosing of antimicrobials in children weighing less than 40 kg should be weight-based. Cefazolin should be dosed at 20 mg/kg to 30 mg/kg IV every 6 hours to 8 hours (up to maximum of 100 mg/kg/d). Metronidazole should be dosed at 30 mg/kg/d IV in four divided doses.

TABLE 3 . Postinjury Antimicrobial Agent Selection and Duration Based Upon Injury Pattern*

Injury	Preferred Agent(s)	Alternate Agent(s)	Duration
Extremity wounds (includes skin, soft tissue, and bone)			
Skin, soft tissue, no open fractures	Cefazolin, 2 g IV q6–8 h†‡	Clindamycin (300–450 mg PO TID or 600 mg IV q8 h)	1–3 d
Skin, soft tissue, with open fractures, exposed bone, or open joints	Cefazolin 2 g IV q6–8 h†‡§	Clindamycin 600 mg IV q8 h	1–3 d
Thoracic wounds			
Penetrating chest injury without esophageal disruption	Cefazolin, 2 g IV q6–8 h†‡	Clindamycin (300–450 mg PO TID or 600 mg IV q8 h)	1 d
Penetrating chest injury with esophageal disruption	Cefazolin 2 g IV q 6–8 h†‡ plus metronidazole 500 mg IV q8–12 h	Ertapenem 1 g IV × 1 dose or moxifloxacin 400 mg IV × 1 dose	1 d after definitive washout
Abdominal wounds			
Penetrating abdominal injury with suspected/known hollow viscus injury and soilage; may apply to rectal/perineal injuries as well	Cefazolin 2 g IV q 6–8 h†‡ plus metronidazole 500 mg IV q8–12 h	Ertapenem 1 g IV × 1 dose or moxifloxacin 400 mg IV × 1 dose	1 d after definitive washout
Maxillofacial and neck wounds			
Open maxillofacial fractures, or maxillofacial fractures with foreign body or fixation device	Cefazolin 2 g IV q6–8 h†‡	Clindamycin 600 mg IV q8 h	1 d
Central nervous system wounds			
Penetrating brain injury	Cefazolin 2 g IV q6–8 h.†‡ Consider adding metronidazole 500 mg IV q8–12 h if gross contamination with organic debris	Ceftriaxone 2 g IV q24 h. Consider adding metronidazole 500 mg IV q8–12 h if gross contamination with organic debris. For penicillin allergic patients, vancomycin 1 g IV q12 h plus ciprofloxacin 400 mg IV q8–12 h	5 d or until CSF leak is closed, whichever is longer
Penetrating spinal cord injury	Cefazolin 2 g IV q6–8 h.†‡ ADD metronidazole 500 mg IV q8–12 h if abdominal cavity is involved	As above. ADD metronidazole 500 mg IV q8–12 h if abdominal cavity is involved	5 d or until CSF leak is closed, whichever is longer
Eye Wounds			
Eye injury, burn or abrasion	Topical: Erythromycin or Bacitracin ophthalmic ointment QID and PRN for symptomatic relief Systemic: No systemic treatment required	Fluoroquinolone 1 drop QID	Until epithelium healed (no fluorescein staining)
Eye injury, penetrating	Levofloxacin 500 mg IV/PO once daily. Before primary repair, no topical agents should be used unless directed by ophthalmology		7 d or until evaluated by a retinal specialist
Burns			
Superficial burns	Topical antimicrobials with twice daily dressing changes (include mafenide acetate‖ or silver sulfadiazine; may alternate between the two), silver-impregnated dressing changed q3–5d, or Biobrane	Silver nitrate solution applied to dressings	Until healed
Deep partial-thickness burns	Topical antimicrobials with twice daily dressing changes, or silver-impregnated dressing changed q3–5d, plus excision and grafting	Silver nitrate solution applied to dressings plus excision and grafting	Until healed or grafted
Full-thickness burns	Topical antimicrobials with twice daily dressing changes plus excision and grafting	Silver nitrate solution applied to dressings plus excision and grafting	Until healed or grafted

TABLE 3 . Postinjury Antimicrobial Agent Selection and Duration Based Upon Injury Pattern* (continued)

Injury	Preferred Agent(s)	Alternate Agent(s)	Duration
Point-of-injury/delayed evacuation¶			
Expected delay to reach surgical care	Moxifloxacin 400 mg PO × 1 dose. Ertapenem 1 g IV or IM if penetrating abdominal injury, shock, or unable to tolerate PO medications	Levofloxacin 500 mg PO × 1 dose. Cefotetan 2 g IV or IM q12 h if penetrating abdominal injury, shock, or unable to tolerate PO medications	Single-dose therapy

IV, intravenous; PO, orally; IM, intramuscularly; TID, three times daily; QID, four times daily; PRN, as needed; CSF, cerebrospinal fluid.

* Postinjury antimicrobial agents are recommended to prevent early posttraumatic infectious complications, including sepsis, secondary to common bacterial flora. Selection is based on narrowest spectrum and duration required to prevent early infections before adequate surgical wound management. This narrow spectrum is selected to avoid selection of resistant bacteria. The antimicrobials listed are not intended for use in established infections, where multidrug-resistant or other nosocomial pathogens may be causing infection.

† Cefazolin may be dosed based on body mass: 1 g if weight ≤80 kg (176 lbs), 2 g if weight 81–160 kg (177–352 lbs), 3 g if weight >160 kg (>352 lbs); doses up to 12 g daily are supported by FDA-approved package insert.

‡ Pediatric dosing: cefazolin, 20–30 mg/kg IV q6–8 h (maximum, 100 mg/kg/d); metronidazole, 7.5 mg/kg IV q6 h; clindamycin 25–40 mg/kg/d IV divided q6–8 h; ertapenem, 15 mg/kg IV or IM q12 (children up to 12 yr) or 20 mg/kg IV or IM once daily (children over 12 yr; maximum, 1 g/d); ceftriaxone, 100 mg/kg/d IV divided q12–24 h (dosing for CNS injury); levofloxacin, 8 mg/kg IV or PO q12 h (levofloxacin is only FDA-approved in children for prophylaxis of inhalational anthrax in children older than 6 mo, but this dose is commonly used for other indications); vancomycin, 60 mg/kg/d IV divided q6 h (dosing for CNS injury); ciprofloxacin, 10 mg/kg IV (or 10–20 mg/kg PO) q12 h.

§ These guidelines do not advocate adding enhanced gram-negative bacteria coverage (i.e., addition of fluoroquinolone or aminoglycoside antimicrobials) in type III fractures.

‖ Mafenide acetate is contraindicated in infants younger than 2 mo.

¶ Postinjury antimicrobial therapy as suggested by the Tactical Combat Casualty Care Committee.

IV. What Duration of Antimicrobials Should be Given to Patients After Combat-Related Injuries?

22. The shortest course of postinjury antimicrobial therapy should be used (**IB**) (Table 3). If multiple wounds are present, the duration of antimicrobials is dictated by the injury pattern requiring the longest duration of therapy. Duration should not be extended for open wounds, drains, or external fixation devices. Wounds should be continually reassessed for evidence of infection and antimicrobials directed specifically at known or empirically suspected infecting pathogens provided if infection is suspected or proven.

Extremity Wounds

23. Antimicrobials should be provided for 1 day to 3 days for all extremity wounds (**IB**).

CNS Wounds

24. Antimicrobials are recommended for 5 days or until cerebrospinal fluid (CSF) leak is closed, whichever time period is longer (**ID**).

Eye, Maxillofacial, and Neck Wounds

25. For penetrating eye injuries, antimicrobials should be provided for a total of 7 days or until a thorough evaluation by a retinal specialist with adequate capabilities has been performed (**IC**).
26. For maxillofacial and neck injuries, 1 day of antimicrobial coverage should be provided (**IC**).

Thoracic and Abdominal Cavity Wounds

27. Thoracic injuries with esophageal injury should also receive a total of 1 day of antimicrobials after definitive operative washout (**IB**).
28. Casualties should receive a total of 1 day of antimicrobials after definitive operative washout for abdominal cavity injuries (**IB**).

Burns

29. Topical antimicrobial agents should be used for burns until wounds are successfully covered with healed skin, whether spontaneously or following successful skin grafting (**IC**).

V. Should Antimicrobials be Redosed Before Next Schedule Dosing Interval if Patients Require Substantial Blood Product Support, Require Large Volume Resuscitation, or Have Severe Acidosis?

30. Redosing of antimicrobials should be performed after large volume blood product resuscitation (1,500–2,000 mL of blood loss) has been completed, regardless of when the last dose of antimicrobial was administered (**IC**).

VI. Should Local Delivery of Antimicrobials Through Topical Application or Beads (Bead Pouches) be Implemented in the Care of Combat-Related Injuries?

31. Local delivery of topical antimicrobials may be provided for extremity infections in the form of antimicrobial beads or pouches as long as the emphasis is still on surgical debridement and irrigation (**IB**).
32. Local delivery of other antimicrobials (other than in burn care), to include powders or soaking of wet to dry dressing with antimicrobials, should not be used routinely (**IB**).

VII. What Vaccines or Other Immunotherapy Should be Provided Postinjury?

Tetanus Toxoid or Immune Globulin

33. Patients who have been previously immunized against tetanus (received 3 or more doses of toxoid) do not require booster dose of vaccine unless it has been more than 5 years since their last dose. They do not require tetanus immune globulin (TIG) (**IB**).
34. Unimmunized patients, and those with unknown vaccination status, should receive TIG and vaccine (with additional doses of vaccine given at 4 weeks and 6 months) postinjury (**IC**).
35. Early surgical debridement and irrigation in addition to postinjury antimicrobials and vaccine may be effective in the prevention of tetanus in the absence of TIG administration (**IID**).

Postsplenectomy Immunization

36. Patients who have had their spleens removed should receive immunization against *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Hemophilus influenza* serotype B (**IB**). Immunization should be provided within 14 days of splenectomy.

C. Debridement and Irrigation

VIII. When Should Irrigation Fluid be Implemented in the Management of Combat-Related Injuries?

37. Wound irrigation should be initiated as soon as clinically possible by appropriately trained personnel (**ID**).

IX. Should Additives Supplement Irrigation Fluid for Combat-Related Injuries?

38. Additives should not be included in standard irrigation fluid as normal saline (or alternately, sterile water or potable water) is adequate (**IB**).

X. What Volume of Fluid Should be Used to Irrigate Wounds Associated With Combat Injuries?

39. Sufficient volume to remove debris should be employed (**IB**). For extremity injuries, standard volumes of 3 L, 6 L, and 9 L should be provided for type I, II, and III fractures, respectively; however, larger volumes might be required for more severe injuries (**IB**).

XI. What Pressure Should be Used to Deliver Irrigation in the Management of Combat-Related Injuries?

40. Irrigation fluid should be delivered at low pressure (5–10 PSI [pounds per square inch] may be delivered by bulb syringe or gravity irrigation) (**IB**).

XII. Should Pre- and/or Postdebridement Bacterial Culture of Combat-Related Wounds be Performed?

41. Clinicians should obtain bacterial cultures only when there are concerns for an ongoing wound infection based upon systemic signs or symptoms of infection, local appearance of wounds, and laboratory or radiographic imaging studies (**IB**).
42. Results from infection control surveillance cultures should not be used for initiation of therapy (**IC**).

XIII. Can Retained Soft Tissue Fragments Remain in a Combat-Related Injury Wound?

43. Casualties with isolated retained deep extremity soft tissue metal fragments meeting certain clinical and radiographic criteria should be treated with a single dose of cefazolin, 2 g IV, without fragment removal (**IB**). Patients should be monitored for evidence of subsequent infection.

D. Surgical Wound Management

XIV. When Should Patients With Combat-Related Injuries Undergo Initial Surgical Management?

44. Patients should be evacuated to surgical care as soon as possible based upon a risk-benefit analysis of the combat environment (**IB**).
45. Penetrating injuries of the eye (**IB**) and spine without neurologic compromise (**IC**) should await surgical debridement until appropriate surgical expertise is available.
46. Foreign material embedded in the brain, which are not readily accessible, should not be removed by non-neurosurgeons (**IB**).
47. All burn injuries should undergo thorough cleansing and debridement, estimation of extent and depth, and coverage with appropriate topical antimicrobial agents within 8 hours of injury (**IC**). Early (within 5 days) excision and grafting is suggested for deep partial-thickness and full-thickness burns (**IA**). This should ideally be performed outside of the combat zone by surgeons with appropriate training and experience.

XV. When Should Combat-Related Wounds be Closed?

48. Wounds, to include open fractures, should not be closed early; typical closure should be performed 3 days to 5 days after injury if there is no evidence of infection (**IB**).

49. For injuries that involve the face or dura, primary closure should be performed (**IB**).
50. For abdominal and thoracic injuries, the skin should not be closed if there is a colon injury or extensive devitalized tissue due to excessive infectious complications (**IB**).
51. Early primary repair of complex or destructive colonic injuries should not be performed especially if associated with massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high-velocity injury, or extensive local tissue damage (**IB**).
52. If the abdomen is left open, the possibility of partial or complete closure should be considered at each subsequent laparotomy (**IB**).
53. Scheduled laparotomies should be performed in this group at 24-hour to 48-hour intervals (**IB**).

XVI. Should External Fixation be Standard for Stabilization of Fracture?

54. Temporary spanning external fixation should be placed for femoral and tibial fractures (**IB**). Use of external fixation in the current conflicts allows stabilization during long evacuations to the United States, easy observation of wounds (over use of plaster), and potentially less chronic infections (over early open reduction and internal fixation).
55. Temporary spanning external fixation or splint immobilization placement with transition to open plate and screw osteosynthesis should be employed for open humerus and forearm fractures after soft tissue stabilization (**IB**).

XVII. Can NPWT be Used in the Management of Combat-Related Wounds?

56. NPWT should be used in the management of open wounds (excluding CNS injuries) to include during aeromedical evacuation of patients (**IB**).
57. Use of intermittent suction or instillation of normal saline in conjunction with NPWT is discouraged in most situations based upon preliminary animal studies (**ID**).
58. Local delivery of antimicrobials using beads or pouches might be effective in combination with NPWT and could be considered (**IID**).

XVIII. Should Supplemental Oxygen be Provided During Transportation of the Wounded to Medical Facilities Outside the Combat Zone?

59. During aeromedical evacuation, supplemental oxygen (to maintain oxygen saturation >92%) may be beneficial in patients with combat-related injuries (**IIC**).

E. Facility Infection Control and Prevention

XIX. What Infection Control and Prevention Measures Should be Implemented in Deployed Medical Treatment Facilities?

60. Basic infection control and prevention measures should be employed at all deployed medical treatment facilities (MTF). These should include hand hygiene, with compliance monitoring. Infection control and prevention should include MTF Commander oversight and emphasis (**IB**).
61. Transmission-based (isolation) precautions should be implemented (**IB**).
62. Cohorting (i.e., physically separating patients expected to be hospitalized for less than 72 hours from those expected to be hospitalized longer) should be used (**IC**).
63. An infection control officer should be assigned to each deployed MTF that provides inpatient care. This officer should have adequate training and experience to lead the infection control program at the MTF.
64. All deployed MTF should practice antimicrobial stewardship (**IC**). Clinical microbiology assets are crucial to antimicrobial stewardship and should be available at MTF which hospitalize patients for more than 72 hours.

INTRODUCTION

Battlefield trauma management emphasizes early delivery of medical care that includes hemorrhage control, hypotensive and hemostatic resuscitation, and administration of antimicrobial therapy with a goal to minimize excess morbidity and mortality.[7–10] Historically, infections have been major complications of combat-related injuries, with an infection rate of 3.9% among 17,726 wounded in the Vietnam War. This rate significantly underestimates the true burden of infection because only data from care provided within the combat zone and during the first 7 days after injury were included.[11] Sepsis, or likely multisystem organ failure, was the third leading overall cause of death and the most common cause of death for those casualties who survived the first 24 hours after injury.[12,13] Studies from the current wars in Iraq and Afghanistan have similarly reported that in those who do die of their wounds, a high incidence die from sepsis or multisystem organ failure secondary to infection.[14,15]

Wounds incurred during combat have resulted in infectious complications to include sepsis and death. These complications continue to be common among recent combat casualties, including those secondary to MDR bacteria such as *Acinetobacter baumannii-calcoaceticus* complex, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, and extended-spectrum β-lactamase-producing organisms such as *Escherichia coli* and *Klebsiella pneumoniae*.[16–19] Severe injuries and admission to an intensive care unit have been shown to be associated with higher infection rates during the current conflicts in Iraq and Afghanistan.[16,20] Gram-negative bacteria infect and colonize casualties in the period immediately after injury, whereas gram-positive bacteria infect and colonize patients during the rehabilitative period.[17–19] Increasing colonization with MDR bacteria throughout the evacuation chain from the combat zone, through Germany, to the United States supports the concept that most MDR bacteria colonization and infection is healthcare-associated.[21–24] The nosocomial spread of MDR bacterial infections throughout the evacuation chain also supports the need for limiting the overuse of broad spectrum antimicrobial agents and emphasizes the need for compliance with infection control measures.

The primary injury patterns associated with combat-related injuries is extremity damage, with increasing rates of maxillofacial and neck injuries and relatively stable number of burn patients during the wars in Iraq and Afghanistan.[25–33] Infection rates have been noted to be ~15% to 25% in the current wars in Iraq and Afghanistan with substantial associated morbidity and mortality.[16,17,34] This rate reaches more than 40% in those wounded who require intensive care unit admission.[35] The goals of combat-related injury care include preventing infection, promoting healing, and restoring function. The *Guidelines for the Prevention of Infection after Combat-Related Injuries* published in 2008 and supporting evidence-based reviews focused on initial stabilization, systemic antimicrobial therapy, wound debridement and irrigation, timely wound closure, and appropriate follow-up.[36–41]

In these guidelines, the previous evidence-based recommendations are updated, using military and civilian data to optimally minimize infections after combat-related trauma. Efforts were made to ensure that these recommendations could be applied across all levels of medical care in a combat zone and could be modified based on the equipment and medical expertise available at each care level. Finally, where necessary, management strategies consider differing evacuation times and the management of personnel not evacuated out of the combat zone (such as local nationals). The utility of antimicrobial agents, debridement and irrigation, surgical wound management, and facility infection control and prevention is emphasized.

PRACTICE GUIDELINES

Practice guidelines are systematically developed statements to assist practitioners and patients in making decisions about appropriate health care for specific clinical circumstances. Attributes of good guidelines include validity, reliability, reproducibility, clinical applicability, clinical flexibility, clarity, multidisciplinary process, review of evidence, and documentation.

METHODOLOGY

Panel Composition

A panel of experts composed of infectious disease (D.R.H., C.K.M., N.G.C., L.C.D., M.A.F., A.D.G., K.E.K., G.J.M., K.P., D.E.S., D.R.T., T.J.W., G.W.W.); surgical specialists, including general surgery/trauma/critical care (G.P.C., W.C.D., J.R.D., B.J.E., J.B.H., J.S.S.), orthopedic surgery (R.C.A., J.H.C., J.C.C., J.R.F., M.E.F., J.R.H., W.T.O.), cardiothoracic surgery (J.M.C.), vascular surgery (T.K.C.), neurosurgery (L.E.M.), ophthalmology (M.H.C.), oral maxillofacial surgery (R.B.B., R.G.H.), otolaryngology (D.K.H.), and burns (L.C.C., E.M.R., J.R.S.); infection control (H.K.C.); preventive medicine (A.R.W.); critical care (K.K.C.); and translational research (J.C.W.) was assembled. US military officers (D.R.H., C.K.M., R.C.A., L.C.C., J.M.C., K.K.C., M.H.C., N.G.C., G.P.C., H.K.C., T.K.C., L.C.D., W.C.D., J.R.D., B.J.E., J.R.F., M.E.F., M.A.F., R.G.H., D.K.H., J.R.H., K.E.K., G.J.M., L.E.M., K.P., E.M.R., D.E.S., T.J.W., A.R.W., G.W.W.), civilian experts (R.B.B., J.H.C., W.T.O., J.R.S., J.S.S., D.R.T., J.C.W.), and two British military medical officers (J.C.C., A.D.G.) were included on the panel. Essentially, all military personnel had experience in Afghanistan and/or Iraq and in caring for casualties from these conflicts outside of the combat zone.

Literature Review and Analysis

Review of the medical literature was performed initially by members of the five review teams based on body system or type of injury. These included teams focused on extremity injuries, CNS injuries, eye, maxillofacial, and neck injuries, thoracic and abdominal cavity injuries, and burn injuries. Literature reviews were performed by searching PubMed for all English language publications relevant to the material of interest from January 2007 through December 2010. All abstracts were reviewed and full-length articles relevant to the subject were pulled for further review of references to be included in literature review and analysis. All articles were then reviewed for populations under study including war-related or civilian trauma, type of study design, and size of study. Focus was on human studies, but key animal studies were included where human data were limited or unavailable. Unpublished research performed by members of the panel was also considered in these recommendations.

Process Overview

In evaluating the evidence regarding the prevention of infections associated with combat-related injury, the panel followed a process used in the development of Infectious Diseases Society of America (IDSA) guidelines. The process included a systematic weighting of the quality of the evidence and the grading of the recommendations using the Grades of Recommendation, Assessment, Development, and Evaluation (GRADE; www.gradeworkinggroup.org) system (Table 1). The first priority was to evaluate articles on military trauma. To supplement this, civilian trauma articles, primarily randomized control trials and then cohort studies, were reviewed. An attempt was made to assign a level to denote both the strength of recommendations and quality of the evidence available to support those recommendations.

Consensus Development Based on Evidence

The review teams evaluated summary documents of key articles and preliminary drafts of their manuscripts in electronic format. Clarification of the quality of evidence and recommendations to present to the entire panel were addressed during these processes. The entire panel met to finalize recommendations and assessments of quality of evidence for the guidelines. All panel members participated in the preparation of the draft guidelines. The contents of the guidelines and the manuscript were reviewed and endorsed by the IDSA Standards and Practice Guideline Committee and IDSA Board of Directors before dissemination.

Guidelines and Conflict of Interest

All panel members complied with the IDSA policy on conflicts of interest, which requires disclosure of any financial or other interest that might be construed as constituting an actual, potential, or apparent conflict. Members of the panel were provided IDSA's conflict of interest disclosure

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statement and were asked to identify ties to companies developing products that might be affected by promulgation of the guideline. Information was requested regarding employment, consultancies, stock ownership, honoraria, research funding, expert testimony, and membership on company advisory committees. The panel made decisions on a case-by-case basis as to whether an individual's role should be limited as a result of a conflict. No limiting conflicts were identified.

Summary of Outcomes Assessed

The information derived from the literature is limited as there are no prospective randomized clinical trials in or out of the combat zone dealing with injuries from the ongoing conflicts in Iraq and Afghanistan for the various clinical questions. Therefore, the data are summarized by military relevant data and then by presenting civilian injury trauma and general trauma studies. Generalizing civilian trauma care data to that of combat trauma care may not be valid because of differences in mechanisms of injury, energy transferred to tissue, time to initial assessment and care, diagnostic capabilities at initial receiving facilities and the austere nature of many of those facilities, and access to and type of medical care systems available. Efforts were also made to ensure that these recommendations could be applied across the different levels of medical care in a combat zone and could be modified based on the equipment and medical expertise available at each level. Finally, management strategies had to incorporate possible differing evacuation times, and the management of personnel not evacuated out of the combat zone.

RECOMMENDATIONS FOR THE PREVENTION OF INFECTIONS ASSOCIATED WITH COMBAT-RELATED INJURIES

A. Initial Care in the Field

I. What Initial Care/Stabilization Should be Provided to the Injured Patient in the Field Before Evacuation to a Medical Care Facility (Medical Treatment Facilities)?

1. Wounds should be bandaged with sterile dressing and fractures stabilized before transportation to higher level of care (**IB**) (Table 2).
2. Dressing covering the eye should provide protection while avoiding producing pressure on the orbit (**IB**). A Fox shield or other such device should be employed.
3. Patients should be transferred to a facility with surgical support as soon as feasible (**IB**) (see recommendation 44).
4. Given the unpredictable nature of casualty evacuation in a combat zone, point-of-injury antimicrobial agents (see recommendation 20) should be provided if evacuation is delayed or expected to be delayed (**IC**).

Evidence Summary

Open wounds should be protected by bandaging with sterile dressings applied to prevent further contamination. Fractures should be splinted to prevent further tissue damage before transporting patients to higher levels of care.[8–10,42] Eye injuries should be protected in a fashion which does not produce pressure on the eye, because pressure placed on an open globe may cause suprachoroidal hemorrhage and irreversible blindness.[43] Use of a Fox shield or improvised field expedient eye cover has been suggested. Dressings applied to open cranial and spinal injuries should provide protection while avoiding producing pressure on the exposed brain or spinal cord. Discussion of the evidence to support recommendations 3 and 4 is included in the evidence summaries for recommendations 44 and 5, respectively.

B. Postinjury Antimicrobials

II. Should Systemic Antimicrobials be Given to Patients With Combat-Related Injuries Immediately Postinjury?

5. Systemic antimicrobials should be administered as soon as possible after injury to prevent early infectious complications, including sepsis, caused by common bacterial flora. Ideally, postinjury antimicrobials should be given within 3 hours of injury (**IB**).

Evidence Summary

Data from previous and current conflicts support early delivery of antimicrobial agents (Murray et al., submitted).[44–46] Although studies among civilian trauma patients do not consistently support earlier delivery of antimicrobial agents, they are supported by various guidelines.[47–52] In addition, animal studies support the premise that earlier antimicrobials can delay the onset of infection and are beneficial.[53–59]

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III. Which Antimicrobials (and What Dosing Regimens) Should be Employed for Postinjury Use?

6. Antimicrobial selection should focus on providing the narrowest spectrum of activity required, providing coverage of expected common bacterial flora. If multiple injuries are present, the antimicrobial agent selection should be based on the narrowest spectrum needed to cover all wound sites/types (**IB**). Postinjury antimicrobials are provided to prevent early infectious complications, including sepsis. These recommended antimicrobials are not meant to treat established infections where nosocomial pathogens, including MDR, may be the infecting agents (Table 3).
7. Selected agents should be dosed to maximize pharmacokinetics and pharmacodynamics. Logistical considerations, including limiting number of agents to be stocked and maintaining sufficient quantities in the combat zone, should also be considered.

Extremity Wounds

8. Cefazolin, 2 g IV every 6 hours to 8 hours, should be used as the antimicrobial of choice in extremity injuries (skin, soft tissue, and/or bone) (**IB**). Clindamycin may be

given as an alternate agent if previous documented anaphylaxis to β-lactam antimicrobials.
9. Enhanced gram-negative coverage should not be employed (**IB**).
10. Addition of penicillin to provide antimicrobial coverage of clostridial gangrene and group A β-hemolytic *Streptococcus* infections is not required (**IC**).

CNS Wounds

11. Cefazolin, 2 g IV every 6 hours to 8 hours, should be employed for CNS injuries (**IB**).
12. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if brain grossly contaminated with organic debris (**ID**).
13. Add metronidazole, 500 mg IV every 8 hours to 12 hours, if spinal cord injury associated with concomitant abdominal cavity penetration (**IC**).

Eye, Maxillofacial, and Neck Wounds

14. For penetrating eye injuries, levofloxacin, 500 mg IV or orally every 24 hours, should be provided (**IB**).
15. For maxillofacial and neck injuries, cefazolin, 2 g IV every 6 hours to 8 hours, should be provided (**IC**). Clindamycin, 600 mg IV every 8 hours, may be used as an alternate (**IC**).

Thoracic and Abdominal Cavity Wounds

16. For thoracic cavity injuries without disruption of the esophagus, cefazolin, 2 g IV every 6 hours to 8 hours, should be used (**IIB**).
17. Cefazolin, 2 g IV every 6 hours to 8 hours, with metronidazole, 500 mg IV every 8 hours to 12 hours, should be provided for penetrating wounds to the abdomen and penetrating wounds to the thorax that result in esophageal injury (**IIB**). Alternate regimens include single-dose ertapenem (1 g IV) or moxifloxacin (400 mg IV) (**IIB**).

Burns

18. Topical antimicrobial agents should be used for burn wounds in conjunction with debridement (**IB**). Silver sulfadiazine cream alternating with mafenide acetate cream is preferred. Debridement may not be feasible at lower levels of care; in this situation, clean, dry dressing should be applied to burn wound until the patient is transferred to a higher level of care.
19. Systemic antimicrobials are not indicated for postinjury therapy (**IC**), or for debridement performed as part of routine wound care (**IB**), unless required for concomitant traumatic injuries. Systemic antimicrobials may be considered for perioperative prophylaxis during excision and grafting procedures (**IC**). Cefazolin, 2 g IV every 6 hours to 8 hours for 24 hours, is sufficient for coverage of skin flora. However, antimicrobial agents effective against *Pseudomonas* should be considered if wounds are grossly colonized or older than 5 days.

Point-of-Injury Antimicrobial Selection

20. Point-of-injury antimicrobials as suggested by the TCCC Committee currently include moxifloxacin, 400 mg orally, if casualty does not have penetrating abdominal trauma, is not in shock, and can take oral medications. In patients who do not meet these criteria, single-dose ertapenem (1 g IV or IM) or cefotetan (2 g IV or IM) every 12 hours has been suggested. IV therapy is preferred over IM.

Pediatric Considerations

21. Children should be treated with the same antimicrobial agents as those suggested for adults, including those topical antimicrobials suggested for burns. Dosing of antimicrobials in children weighing less than 40 kg should be weight-based. Cefazolin should be dosed at 20 mg/kg to 30 mg/kg IV every 6 hours to 8 hours (up to maximum of 100 mg/kg/d). Metronidazole should be dosed at 30 mg/kg/d IV in four divided doses.

Evidence Summary

The antimicrobials of choice were selected to maximize pharmacokinetics and pharmacodynamics for patients with multiple injuries while minimizing the number of agents needed to be stocked and employed in the combat zone. In addition, focus was placed on recommending antimicrobial agents with the most limited spectrum needed for postinjury use to avoid driving the selection of MDR bacteria. Overall, the agents selected should include coverage of all injury types that a particular patient has. Use of high-dose cefazolin is based on pharmacokinetic studies of dosing based on patient weight.[60–62] Dosing of metronidazole at intervals more than every 8 hours is also supported by recent data.[63] In addition to the management of coalition and local adult patients, host-nation pediatric patients constitute a large percentage of those receiving care in the combat hospitals with infections being a common complication.[64–67]

Extremity Wounds

Postinjury antimicrobial agent selection is primarily based on retrospective studies and expert opinion, with data typically focused on more severe extremity injuries, notably type III fractures.[47–49,68–73] Of wounds not needing surgical evacuation in a combat zone, a single study revealed the overall importance of wound irrigation over systemic antimicrobials.[74] High-dose cefazolin was selected in this guideline because of concerns of underdosing wounded personnel who weigh more than 70 kg and low serum concentrations of drug with blood loss.[75] The package insert indicates that up to 12 g/d of cefazolin has been used.[60,61,76] A recommendation against adding enhanced gram-negative coverage was based on the lack of clear data documenting the benefit of this practice and concerns that adding a fluoroquinolone or aminoglycoside might increase selection of subsequent nosocomial MDR pathogens. In addition, no single aminoglycoside has been identified that could potentially cover all the MDR bacteria currently being recovered subsequently in the care of combat casualties, and all these agents carry the concern for potential renal toxicity in under-resuscitated patients who might sustain hypovolemic renal injury.[77–80] Clindamycin was selected as an alternative therapy based upon controlled trials revealing efficacy, especially in type I and II fractures.[73,81]

The incidence of gas gangrene and streptococcal infections after injury has remained exceedingly low during the prolonged conflicts in Afghanistan and Iraq. This is likely secondary to aggressive surgical management with delayed primary closure of wounds. In addition, both *Clostridium perfringens* and *Streptococcus pyogenes* are likely covered with the antimicrobials currently provided after combat-related injuries, and thus the addition of penicillin should not be given.[47,68,69,82–88]

CNS Wounds

Several recent review articles have summarized data from civilian and military traumatic casualties resulting in penetrating brain injury and have recommended the use of postinjury antimicrobials for the prevention of infection.[89,90] The data supporting these recommendations are based on retrospective reviews and expert opinion and do not support a standard treatment regimen or duration. For penetrating injuries to the spine, multiple reports have shown a 0% to 32% infectious complication rate and varied postinjury antimicrobial usage.[91–97]

Eye, Maxillofacial, and Neck Wounds

Given the excellent pharmacokinetics and effective spectrum of coverage of the newer fluoroquinolone agents, administration of systemic levofloxacin or moxifloxacin should be sufficient to prevent endophthalmitis after traumatic (penetrating) eye injury.[98–100] Retrospective review has demonstrated low rates of endophthalmitis with use of these agents.[101]

Antimicrobial therapy with ampicillin, penicillin, and cephalosporins has been used effectively in maxillofacial and neck combat injuries, but the organisms causing infection, dosing, duration of therapy, and definition of infection are poorly described.[102,103] However, randomized controlled trials of antimicrobial prophylaxis of infection for contaminated head and neck surgery (nontrauma patients) show a 77% to 79% reduction in infection compared with placebo.[104,105] Therefore, postinjury antimicrobial therapy of the contaminated injuries of combat trauma is recommended. Recommended agents are based on data from the same nontrauma population and include high-dose cefazolin, 2 g IV every 6 hours to 8 hours.[106] This higher dose is preferred as lower doses did not seem to be as effective.[107] Alternate use of clindamycin (600 mg IV every 8 hours) is also supported by the noncombat trauma literature.[108,109]

Thoracic and Abdominal Cavity Wounds

Postinjury antimicrobial selection for thoracic and abdominal cavity trauma is based on trauma data from the civilian community.[110–114] Use of ertapenem is based on its perioperative use in elective colorectal surgery.[115] Moxifloxacin has been demonstrated to have comparable efficacy to combination therapies in recent studies of complicated intra-abdominal infections.[116–119]

Burns

Topical antimicrobial therapy is currently the standard in postburn care.[120] Systemic antimicrobial agents are not recommended for debridement performed as part of routine wound care but have been used for perioperative prophylaxis during excision and grafting procedures, especially in patients with larger burns, although the data for this practice are inconclusive. Early studies documented a significant incidence of transient bacteremia associated with wound manipulation,[121] but a more recent evaluation showed this incidence to be much reduced.[122] Antimicrobial administration has been found to reduce the incidence of this transient bacteremia but did not affect outcomes.[123] A recently published study by Ramos et al.[124] found that the use of systemic perioperative antimicrobial administration for patients undergoing grafting of deep burns was associated with improved autograft survival. However, the study had several limitations, including a small sample size, and a more extensive follow-up study will be required. Because of the limited evidence, controversy on this topic exists, and burn units vary widely in their practices of providing perioperative antimicrobial prophylaxis.[125,126] Although the data are inconclusive, the clinician may consider the use of perioperative systemic antimicrobials for excision and grafting procedures.

Point-of-Injury Antimicrobial Selection

A panel of military trauma experts on point-of-injury care (TCCC Committee) have recommended oral moxifloxacin and intravenous/intramuscular cefotetan or ertapenem as point-of-injury antimicrobials.[8–10,127] Selection of point-of-injury field antimicrobials is based on three criteria: (1) activity against the expected infecting pathogens for the body part injured, (2) stability in the field environment, and (3) ease of delivery (dosing interval and volume of infusion) on the battlefield with minimal adverse events.[9,10,127,128] A recent study evaluating point-of-injury antimicrobials by US Army Rangers did not seem to show clear infection prevention benefit, although the numbers were small. Of note, no increases in colonization or infection with MDR bacteria were noted, nor were medication toxicities reported. There are clear arguments for choosing agents with much narrower antibacterial spectrums of activity; however, it seems the antimicrobials recommended by the TCCC Committee are not causing harm and may be beneficial. TCCC recommendations include use of IV or IM ertapenem or cefotetan for point-of-injury antimicrobials in those wounded unable to take oral agents.[8–10] Although TCCC Committee has also made recommendations for the use of the intraosseous (IO) delivery route for fluid and analgesic therapy, IO delivery of antimicrobials has not been systematically studied in military populations or trauma patients.[129,130] In animal studies, those antimicrobials that are highly protein bound were associated with lower serum concentrations with IO delivery compared with IV delivery.[131] Both cefazolin and ertapenem are highly protein bound antimicrobials. Although IM delivery has also not been studied in military or trauma patient populations, both cefazolin and ertapenem are approved by the Food and Drug Administration for use by this route.

Pediatric Considerations

Pediatric trauma is a common occurrence in the combat theater, and children are frequently cared for in deployed medical settings. The appropriate choices of antimicrobial

agents for the prevention of trauma-related infection in children are essentially identical to those for adults. Accurate weight-based dosing of these drugs is critical as the pharmacokinetics of these medications in the young child often results in higher dose-per-weight and more frequent dosing requirements. In general, adult dosing of antimicrobials should be used in children weighing 40 kg or more, as weight-based dosing about this can result in doses exceeding the maximum adult dosage. Neonates younger than 28 days, or those weighing less than 2 kg, have significantly different metabolism and clearance of most antimicrobials, and different regimens should be used.

The doses of the most commonly used antimicrobial agents include cefazolin (20–30 mg/kg IV every 6–8 hour, up to a maximum dose of 100 mg/kg/d) and metronidazole (30 mg/kg/d IV, divided into 4 daily doses). Ertapenem has been approved for use in children older than 3 months; however, once daily dosing is inadequate. The recommended dose is 15 mg/kg IV or IM every 12 hours for children through 12 years (for children older than 12 years, the dose is 20 mg/kg once daily, with a maximum dose of 1 g).

Although limited data are available on the safety and dosage of moxifloxacin in children, ciprofloxacin is a well-studied and safe option in pediatric. Ciprofloxacin (10 mg/kg IV every 12 hours) or levofloxacin (8 mg/kg IV every 12 hours) in combination with metronidazole is a reasonable choice for postinjury therapy of penetrating abdominal injuries in children. Pediatric dosing for other antimicrobials recommended in these guidelines include clindamycin 25 mg/kg/d to 40 mg/kg/d IV divided into 6- to 8-hour dosing. Antimicrobial dosing of the alternate agents for CNS trauma includes vancomycin 60 mg/kg/d divided into 6- to 8-hour dosing and ceftriaxone 100 mg/kg/d IV given in every 12 hours or once daily.

The use of topical antimicrobials in pediatric burns is similar to that used in adults, with the exception that mafenide acetate should be avoided in neonates because of the risk of kernicterus association with sulfonamides.

IV. What Duration of Antimicrobials Should be Given to Patients After Combat-Related Injuries?

22. The shortest course of postinjury antimicrobial therapy should be used (**IB**) (Table 3). If multiple wounds are present, the duration of antimicrobials is dictated by the injury pattern requiring the longest duration of therapy. Duration should not be extended for open wounds, drains, or external fixation devices. Wounds should be continually reassessed for evidence of infection and antimicrobials directed specifically at known or empirically suspected infecting pathogens provided if infection is suspected or proven.

Extremity Wounds

23. Antimicrobials should be provided for 1 day to 3 days for all extremity wounds (**IB**).

CNS Wounds

24. Antimicrobials are recommended for 5 days or until CSF leak is closed, whichever time period is longer (**ID**).

Eye, Maxillofacial, and Neck Wounds

25. For penetrating eye injuries, antimicrobials should be provided for a total of 7 days or until a thorough evaluation by a retinal specialist with adequate capabilities has been performed (**IC**).
26. For maxillofacial and neck injuries, 1 day of antimicrobial coverage should be provided (**IC**).

Thoracic and Abdominal Cavity Wounds

27. Thoracic injuries with esophageal injury should also receive a total of 1 day of antimicrobials after definitive operative washout (**IB**).
28. Casualties should receive a total of 1 day of antimicrobials after definitive operative washout for abdominal cavity injuries (**IB**).

Burns

29. Topical antimicrobial agents should be used for burns until wounds are successfully covered with healed skin, whether spontaneously or following successful skin grafting (**IC**).

Evidence Summary

Based upon the civilian trauma literature, existing military and civilian guidelines, and the high prevalence of (presumed nosocomial) MDR bacterial infections being reported among casualties from Iraq and Afghanistan and the risk of prolonged antimicrobial therapy in increasing rates of nosocomial infections, short courses of postinjury antimicrobial therapy should be used.

Extremity Wounds

Postinjury antimicrobial therapy should be given for at least 24 hours. Civilian data focused on severe (type III) extremity fractures support continuing therapy for 1 day to 3 days with reassessment of wounds. Antimicrobial agents should only be continued for ongoing infection and then directed at the bacteria's specific resistance profile instead of the prevention focus of initial antimicrobials.[49,51,68,69,132–136]

CNS Wounds

There are no controlled trials identifying the optimal duration of postinjury antimicrobial therapy. A previous review has recommended 5 days for penetrating craniocerebral injury with retained organic material.[89] For penetrating injuries of the spine, one review suggested antimicrobial use for a minimum of 48 hours with extension to 7 days if the alimentary tract was violated.[93] A recent review of traumatic brain and spinal cord injury from the current conflicts in Iraq and Afghanistan revealed baseline rates of meningitis consistent with previous wars but noted a three times higher incidence of meningitis in patients with CSF leaks.[137] Based on the available literature, antimicrobial therapy should be continued for 5 days or until CSF leak control has occurred.

With ventriculostomy placement, it is common practice by many neurosurgeons to continue postinjury antimicrobials until final removal of these devices. Data to support or discourage this practice are not currently available.

Eye, Maxillofacial, or Neck Wounds

No studies in combat ocular trauma patients have been performed to define duration of postinjury antimicrobial therapy. Traumatic endophthalmitis is generally a rapid-onset, fulminant process that creates substantial ocular morbidity.[138] Treatment in these cases generally requires a combination of intravitreal antimicrobials and vitrectomy surgery.[139] Because vitreoretinal capabilities are not available or advised until casualties reach tertiary care outside the combat zone, it is recommended that systemic antimicrobial therapy continues until the patient arrives where surgical management would be possible in the event of endophthalmitis. In the event of delayed evacuation, no less than a 7-day course of treatment is recommended.[101]

No studies in combat trauma victims exist to best define duration of therapy in maxillofacial or neck injury. However, both recent and previous studies of mandibular fractures and contaminated head and neck cases with similar outcomes have all concluded antimicrobial therapy in excess of 24 hours perioperatively do not seem to reduce wound infections.[140–145] Thus, postinjury antimicrobial therapy should be discontinued 24 hours postoperatively.

Thoracic and Abdominal Cavity Wounds

With prompt surgical management, postinjury antimicrobial therapy can be limited to 1 day in thoracic and abdominal cavity injuries.[110,146,147]

Burns

There are no existing studies that define the optimal duration of topical antimicrobial therapy for burn wounds. It is common practice at the US Army Institute of Surgical Research burn center for topical antimicrobial agents to be used until wounds are successfully covered with healed skin, whether by spontaneous healing or after successful skin grafting.

V. Should Antimicrobials be Redosed Before Next Schedule Dosing Interval if Patients Require Substantial Blood Product Support, Require Large Volume Resuscitation, or Have Severe Acidosis?

30. Redosing of antimicrobials should be performed after large volume blood product resuscitation (1,500–2,000 mL of blood loss) has been completed, regardless of when the last dose of antimicrobial was administered (**IC**).

Evidence Summary

Large volume resuscitation with IV fluids and blood products may result in hemodilution of postinjury antimicrobial therapy. Redosing of antimicrobial agents after large volume resuscitation or blood loss (estimated at 1,500–2,000 mL of blood loss) is supported by the civilian medical literature.[62,148–151]

VI. Should Local Delivery of Antimicrobials Through Topical Application or Beads (Bead Pouches) be Implemented in the Care of Combat-Related Injuries?

31. Local delivery of topical antimicrobials may be provided for extremity infections in the form of antimicrobial beads or pouches as long as the emphasis is still on surgical debridement and irrigation (**IB**).
32. Local delivery of other antimicrobials (other than in burn care), to include powders or soaking of wet to dry dressing with antimicrobials, should not be used routinely (**IB**).

Evidence Summary

Local delivery of topical antimicrobials has been used in the surgical treatment of bony and orthopedic device-related infections for several decades. Use of local wound therapy in the form of antimicrobial beads or pouches is used adjunctively and is not a substitute for good surgical debridement and irrigation. Local antimicrobial beads may be used even if NPWT is used. However, data do not support the local delivery of other antimicrobials to include powder or soaking of wet to dry dressing with antimicrobials.[152–168] Direct application of antimicrobials to the brain or spinal cord is contraindicated in the absence of the ability to monitor serum and spinal fluid antimicrobial levels.

VII. What Vaccines or Other Immunotherapy Should be Provided Postinjury?

Tetanus Toxoid or Immune Globulin

33. Patients who have been previously immunized against tetanus (received 3 or more doses of toxoid) do not require booster dose of vaccine unless it has been more than 5 years since their last dose. They do not require TIG (**IB**).
34. Unimmunized patients, and those with unknown vaccination status, should receive TIG and vaccine (with additional doses of vaccine given at 4 weeks and 6 months) postinjury (**IC**).
35. Early surgical debridement and irrigation, in addition to postinjury antimicrobials and vaccine may be effective in the prevention of tetanus in the absence of TIG administration (**IID**).

Postsplenectomy Immunization

36. Patients who have had their spleens removed should receive immunization against *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Hemophilus influenza* serotype B (**IB**). Immunization should be provided within 14 days of splenectomy.

Evidence Summary

Provision of tetanus immunotherapy to prevent infections in contaminated wounds has been the standard of care for decades. Treatment with vaccine or immune globulin is based on whether patient has previously received adequate immunization (3 or more doses of tetanus toxoid). However,

the only cases seen to date within the combat zone have been in Afghan and Pakistani civilians managed in military hospitals after the 2005 Pakistan earthquakes. These cases presented days after their traumatic injuries. In the past several years, a shortage of TIG has resulted in numerous patients being managed without TIG immune therapy. That tetanus has not been reported in this group has been postulated to be due to the effectiveness of early wound care and postinjury antimicrobials (personal communication, Dr. Andrew Green).

Spleen removal places patients at risk for overwhelming postsplenectomy sepsis from encapsulated bacteria, especially *Streptococcus pneumoniae*. Because of this risk, immunization with pneumococcal vaccine has been provided, as has meningococcal and *Hemophilus* vaccine, albeit at a lower rate. Ideal timing of immunization postsplenectomy is not clear, although two studies of immunologic response to vaccine in this setting support giving vaccine at 14 days post removal.[169,170] Immunization with pneumococcal (and other vaccines) vaccine has typically given by trauma surgeons from immediately postoperatively to up to 6 weeks.[171]

C. Debridement and Irrigation

VIII. When Should Irrigation Fluid be Implemented in the Management of Combat-Related Injuries?

37. Wound irrigation should be initiated as soon as clinically possible by appropriately trained personnel (**ID**).

Evidence Summary

Wound irrigation should be initiated as soon as clinically possible by appropriately trained personnel based upon a small military study and animal data.[74,172]

IX. Should Additives Supplement Irrigation Fluid for Combat-Related Injuries?

38. Additives should not be included in standard irrigation fluid as normal saline (or alternately, sterile water or potable water) is adequate (**IB**).

Evidence Summary

Additives should not be included in standard irrigation fluid as normal saline (including sterile water or potable water) is adequate, and additives often are associated with increased tissue damage and subsequent bacterial rebound in the wounds of animal studies.[132,173–179] A large clinical trial looking at irrigant additives for extremity injuries is underway which might modify this recommendation in the future.[174]

X. What Volume of Fluid Should be Used to Irrigate Wounds Associated With Combat Injuries?

39. Sufficient volume to remove debris should be employed (**IB**). For extremity injuries, standard volumes of 3 L, 6 L, and 9 L should be provided for type I, II, and III fractures, respectively; however, larger volumes might be required for more severe injuries (**IB**).

Evidence Summary

The volume of fluid sufficient to fully irrigate most wounds is unknown. Standard volumes of 3 L, 6 L, and 9 L have been suggested and promoted for irrigation of type I, II, and III fractures, respectively.[173,179] However, as the size of wounds varies, even among these defined categories, selection of irrigant volume must be based on that required for the adequate decontamination of any unique wound.

XI. What Pressure Should be Used to Deliver Irrigation in the Management of Combat-Related Injuries?

40. Irrigation fluid should be delivered at low pressure (5–10 PSI, may be delivered by bulb syringe or gravity irrigation) (**IB**).

Evidence Summary

Irrigation fluid pressure should be low pressure (5–10 PSI) as higher pressure irrigation likely damages tissue and possibly push contamination further into wound, resulting in rebound increase in bacterial contamination at 24 hours to 48 hours.[132,174] It is anticipated that the FLOW multicenter, randomized trial will clarify the role of low versus high pressure in extremity injuries.[174]

AQ: 5

XII. Should Pre- and/or Postdebridement Bacterial Culture of Combat-Related Wounds be Performed?

41. Clinicians should obtain bacterial cultures only when there are concerns for an ongoing wound infection based upon systemic signs or symptoms of infection, local appearance of wounds, and laboratory or radiographic imaging studies (**IB**).
42. Results from infection control surveillance cultures should not be used for initiation of therapy (**IC**).

Evidence Summary

Routine sampling of clinically uninfected wounds is not supported as a method to select postinjury or empirical antimicrobial therapy. Clinicians should obtain bacterial cultures only when there are concerns for an ongoing wound infection based upon systemic signs or symptoms of infection, local appearance of wound, and laboratory or radiographic imaging studies.[17–19,46,49,69,180–197] Infection control surveillance cultures should not be used for initiation of therapy as that would expose patients to unnecessary antimicrobials with potential excess toxicity and selection for MDR bacteria.

XIII. Can Retained Soft Tissue Fragments Remain in a Combat-Related Injury Wound?

43. Casualties with isolated retained deep extremity soft tissue metal fragments meeting certain clinical and radiographic criteria should be treated with a single dose of cefazolin, 2 g IV, without fragment removal (**IB**). Patients should be monitored for evidence of subsequent infection.

Evidence Summary

Combat injuries often result in retained fragments of metallic or other materials within the soft tissues which are too deep or too numerous to easily remove without the removal procedure itself creating further morbidity. In the absence of infection or concerns of complications (based on location), it is not necessary to remove all of these foreign bodies. Criteria for observation of small retained fragments include X-ray confirmation revealing no bone involvement, no vascular involvement, and no break of pleura or peritoneum, wound entry/exit lesions less than 2 cm in maximal dimension, and no signs of infection.[198–212] Although previous studies have used 5 days of therapy, response to single-dose therapy has been described in the current conflicts and is likely adequate based upon civilian extremity management.

D. Surgical Wound Management

XIV. When Should Patients With Combat-Related Injuries Undergo Initial Surgical management?

44. Patients should be evacuated to surgical care as soon as possible based upon a risk-benefit analysis of the combat environment (**IB**).
45. Penetrating injuries of the eye (**IB**) and spine without neurologic compromise (**IC**) should await surgical debridement until appropriate surgical expertise is available.
46. Foreign material embedded in the brain, which are not readily accessible, should not be removed by non-neurosurgeons (**IB**).
47. All burn injuries should undergo thorough cleansing and debridement, estimation of extent and depth, and coverage with appropriate topical antimicrobial agents within 8 hours of injury (**IC**). Early (within 5 days) excision and grafting is suggested for deep partial-thickness and full-thickness burns (**IA**). This should ideally be performed outside of the combat zone by surgeons with appropriate training and experience.

Evidence Summary

Patients should be evacuated to surgical care as soon as possible based upon a thorough risk benefit analysis of the combat environment.[11,44,46,49,50,69,86,134,185–188,196,213–222] An interesting study of high-energy lower extremity trauma indicated that care at a definitive trauma center was vital.[52] Eye and spine injuries without neurologic compromise should await surgical debridement until appropriate surgical expertise is available; cerebral foreign bodies should remain if removal would cause excess damage.[223–229]

Extremity Wounds

Data assessing outcomes based on time to procedures are limited for combat casualties, although most of the data indicate delayed interventions are associated with increased infection.[44,46,214,230] Civilian guidelines recommend that rapid surgical debridement is the primary treatment and antimicrobials are adjuvant therapy for infection prophylaxis in open fracture management.[48,132,215] The civilian literature, however, is mixed on the benefit of early surgical intervention.[49,50,196,217–222] A recent study of 315 severe high-energy extremity injuries revealed that time to debridement was not associated with infection (<5 hours, 28% infected [93 patients]; 5–10 hours, 29.1% infected [86 patients]; >10 hours, 25.8% infected [128 patients]).[52] Interestingly this study indicated that time to a definitive trauma center was the most important factor on decreasing infection rate.

CNS Wounds

Historically, extensive debridement of retained material had been recommended for penetrating brain injury; however, recent reviews have shown improved preservation of brain function with less aggressive surgical debridement.[223–229] Thus, current management is to remove only easily accessible foreign material and grossly devitalized tissue. In penetrating spinal injuries, retained bullets have not been shown to be a significant risk factor for infectious complications unless the injury is associated with gross contamination or a tract exists from the peritoneal cavity to the spinal canal.[93] In the latter instances, exploration and low pressure irrigation of the wound are recommended. In patients with declining neurologic function, early removal of bone fragments or foreign bodies causing compression of neurologic structures is recommended to prevent further neurologic compromise.

Eye, Maxillofacial, and Neck Wounds

Rapid evacuation and treatment of the maxillofacial and neck wounds, to include the use of antimicrobials resulted in a decrease in mortality from 40% in World War II to 1.3% during the Korean War.[231,232] One factor attributed to the low incidence of endophthalmitis during the current conflicts has been the early primary closure of open globes (within 6 hours).[101] Given the low rate of infection, the current treatment paradigm is recommended.

Thoracic and Abdominal Cavity Wounds

Thoracic injuries requiring tube thoracostomy will, in many combat related cases, require urgent placement in the field. In one study in a civilian trauma setting, prehospital thoracostomy performed by a physician at the accident scene was determined to be safe but had only a nonsignificant decrement in infected hemothoraces.[233] Placement by more experienced providers was associated with fewer complications in another series.[234] Reevaluation and early evacuation of residual clot should be performed to minimize development of infected hematoma and empyema.[235]

Prompt surgical intervention has been the standard in combat wounds to the abdomen since World War I. Regarding closure of the skin, a number of series of civilian abdominal and colonic injuries, associated with fewer high-velocity penetrating injuries, primary skin closure has been advocated with good success.[236,237]

Controversy in abdominal trauma currently revolves around the timing of closure of the abdominal fascia. Severely injured, combat or noncombat-related abdominal injuries have improved outcomes with "damage control surgery" consisting of an immediate abbreviated laparotomy with goals of hemostasis, limitation of contamination through closure or resection of bowel perforations, delayed bowel anastomoses or ostomies, and wound packing, all in an effort

to provide rapid restoration of physiologic parameters. Delayed closure and use of vacuum pack technique with subsequent definitive surgery is recommended.[238–244]

Burns

Early burn excision, within 5 days of injury, seems to improve survival in patients without inhalation injuries.[245–247]

XV. When Should Combat-Related Wounds be Closed?

48. Wounds, to include open fractures, should not be closed early; typical closure should be performed 3 days to 5 days after injury if there is no evidence of infection (**IB**).
49. For injuries that involve the face or dura, primary closure should be performed (**IB**).
50. For abdominal and thoracic injuries, the skin should not be closed if there is a colon injury or extensive devitalized tissue due to excessive infectious complications (**IB**).
51. Early primary repair of complex or destructive colonic injuries should not be performed especially if associated with massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high-velocity injury, or extensive local tissue damage (**IB**).
52. If the abdomen is left open, the possibility of partial or complete closure should be considered at each subsequent laparotomy (**IB**).
53. Scheduled laparotomies should be performed in this group at 24- to 48-hour intervals (**IB**).

Evidence Summary

Extremity Wounds

Based upon historical war wound management, early closure of open fracture wounds should not be performed and closure should not be performed until 3 days to 5 days after injury.[173,248–252] Definitive bone coverage should performed as soon as feasible after definitive stabilization.[46,253]

CNS Wounds

It is important to close the injury site as quickly as possible, but with penetrating CNS trauma there is often inadequate dura available. An autologous vascularized pericranial tissue graft or commercially available dural substitute can be used successfully in these instances. Cranialization of any violated sinuses and watertight dural and skin closure should follow adequate debridement. In patients who have undergone aggressive cranial decompression after severe blunt or penetrating head injury, the removed bone flap should be discarded if the patient will ultimately be evacuated to a location where custom prosthetic implants are available.[254] Where prosthetic implants are not available (e.g., for nonevacuated local nationals), removed skull fragments should be thoroughly washed and then either replaced or inserted into the abdominal wall fat as a temporary storage location. If the deployed location has a -70°C freezer, this is another option for storage.

Eye, Maxillofacial, and Neck Wounds

For injuries that involve the face, primary closure should be performed.[255]

Thoracic and Abdominal Cavity Wounds

For abdominal injuries, skin should not be closed if there is a colon injury or extensive devitalized tissue due to excessive infectious complications. Early primary repair of complex or destructive colonic injuries should not be performed especially if associated with massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high-velocity injury, or extensive local tissue damage.[238,240,256]

XVI. Should External Fixation be Standard for Stabilization of Fracture?

54. Temporary spanning external fixation should be placed for femoral and tibial fractures (**IB**). Use of external fixation in the current conflicts allows stabilization during long evacuations to the United States, easy observation of wounds (over use of plaster), and potentially less chronic infections (over early open reduction and internal fixation).
55. Temporary spanning external fixation or splint immobilization placement with transition to open plate and screw osteosynthesis should be employed for open humerus and forearm fractures after soft tissue stabilization (**IB**).

Evidence Summary

Staged fixation in combat injuries has emerged as the strategy of choice in this conflict.[37] Temporary external fixation has been commonly used as a bridge to definitive fixation with few significant complications.[257] Although a few selected cases of low-energy injuries have been safely internally fixed in the combat zone, it is still considered "ill-advised" in combat-related injuries.[257,258] The use of plaster splints has been recommended and might be useful with rapid evacuations to more definitive orthopedic expertise.[46,230,259]

XVII. Can NPWT be Used in the Management of Combat-Related Wounds?

56. NPWT should be used in the management of open wounds (excluding CNS injuries) to include during aeromedical evacuation of patients (**IB**).
57. Use of intermittent suction or instillation of normal saline in conjunction with NPWT is discouraged in most situations based upon preliminary animal studies (**ID**).
58. Local delivery of antimicrobials using beads or pouches might be effective in combination with NPWT and could be considered (**IID**).

Evidence Summary

NPWT is effective in the management of open wounds (excluding CNS injuries) to include during aeromedical evacuation of patients out of the combat zone. Battery power may be a limitation to its use on longer transports (>8–10 hours).[25,162,173,253,260–265] Intermittent suction or instillation therapy of normal saline should not be implemented based upon preliminary animal studies because of concern for tissue damage (personal communication, Dr. Joseph Wenke). In severe injuries that cannot undergo adequate surgical debride-

ment (e.g., extensive high bilateral lower extremity injuries with perineum involvement secondary to explosive trauma), where the possible risk of local tissue damage from antiseptics is outweighed by preventing or controlling infection, anecdotal success with topical antiseptics (e.g., Dakin's) in conjunction with NPWT has been reported (personal communication, Dr. Romney Andersen).

XVIII. Should Supplemental Oxygen be Provided During Transportation of the Wounded to Medical Facilities Outside the Combat Zone?

59. During aeromedical evacuation, supplemental oxygen (to maintain oxygen saturation > 92%) may be beneficial in patients with combat-related injuries (**IIC**).

Evidence Summary

The role of oxygen as therapy has been evaluated and pursued in previous wars especially in association with gas gangrene.[266–269] More recently, there has been an ongoing concern regarding low oxygenation level in patients with wounds that occur with long-distance air evacuation from the combat zone to Germany and from Germany to the United States. Preliminary animal studies show decreased bacterial burden when hypoxia is treated with supplemental oxygen to maintain an oxygen saturation of more than 93% (personal communication, Dr. Warren Dorlac). In addition, prospective (civilian, nontrauma) studies have shown mixed results of the use of oxygen supplementation in preventing postsurgical infectious after abdominal and pelvic surgeries, although these studies were not associated with hypoxia induced by elevation.[270–272]

E. Facility Infection Control and Prevention

XIX. What Infection Control and Prevention Measures Should be Implemented in Deployed Medical Treatment Facilities?

60. Basic infection control and prevention measures should be employed at all deployed MTF. These should include hand hygiene, with compliance monitoring. Infection control and prevention should include MTF Commander oversight and emphasis (**IB**).
61. Transmission-based (isolation) precautions should be implemented (*IB*).
62. Cohorting (i.e., physically separating patients expected to be hospitalized for less than 72 hours from those expected to be hospitalized longer) should be used (**IC**).
63. An infection control officer should be assigned to each deployed MTF that provides inpatient care. This officer should have adequate training and experience to lead the infection control program at the MTF.
64. All deployed MTF should practice antimicrobial stewardship (**IC**). Clinical microbiology assets are crucial to antimicrobial stewardship and should be available at MTF which hospitalize patients for more than 72 hours.

Evidence Summary

Infection control and prevention has developed as critical practice to prevent or decrease healthcare-associated infections in MTF. National (civilian) guidelines have been developed by the Centers for Disease Control and Prevention and by other national professional organizations (e.g., IDSA; Society for Healthcare Epidemiology of America [SHEA]; and Association for Professionals in Infection Control and Epidemiology [APIC]). Following the consensus conference to develop our initial guidelines (i.e., *Guidelines for the Prevention of Infection after Combat-Related Injuries*),[38] a review of the deployed MTF in Iraq, Afghanistan, and Kuwait was conducted to assess infection control and prevention challenges and practice in the combat zone.[273] This review led to recommendations for improvement and development of a short course for infection control officers who were to be assigned to a deployed MTF.[273–275]

RESEARCH GAPS

Most of the recommendations included in these guidelines are based on civilian trauma clinical research, retrospective review of combat trauma interventions and outcome, animal research and expert opinion. Research to better answer each of the 19 questions posed in these guidelines is needed. Research gaps include but are not limited to:

- Identifying the best timing of initiation of postinjury antimicrobial therapy.
- Establishing the shortest effective duration needed for postinjury antimicrobial therapy.
- Identifying the best postinjury antimicrobial agents.
- Further evaluation of topical wound therapies, including irrigants.
- Evaluating the role of topical decolonization/cleansing to prevent MDR infections.

In addition, other areas of research could potentially impact efforts to prevent infections in the combat-injured population. These include research into the ecology of wounds (microbiome and biofilm development), the pathophysiology and host immune response associated with when and if infections develop, and development of new diagnostic, prevention, and treatment technologies and strategies. Ongoing epidemiology is also vital to quickly identify changing wounding and infection patterns and the emergence of new etiologic agents.

A better understanding of the wound microbiome and its natural evolution in both injuries which do and do not get infected could better guide care and improve outcomes. Understanding the development and role biofilms play in both acute and chronic wounds and how these interact with the host's immune response could also guide diagnostic and targeted treatment strategies. Diagnostic testing advances in conjunction with enhanced knowledge of the wound microbiome, biofilms, and immune response could identify which patients need antimicrobial therapy, whether this could be local or systemic, and when a wound might be successfully closed. The diagnostic use of inflammatory markers and cytokines is currently being examined as a tool to identify when wounds can be closed without further infectious complications.[276–281]

Invasive fungal infections have recently emerged as an important infectious complication of severe combat injury. Based upon data to date, patients with large bilateral lower extremity injuries typically in lush vegetative areas on dismounted patrol requiring large volume blood product support have been noted to have increased reports of fungal infections, which is consistent with some farm trauma studies.[81,282–284] However at this time, there are inadequate data to determine the role empiric antifungal therapy or tissue characterization techniques with culture or histology. Research is urgently needed to better define the risk factors associated with these infections and to identify potential interventions to prevent this life-threatening complication of combat-related injuries.

PERFORMANCE MEASURES

Performance measures are often used with guidelines to measure effectiveness or benefits of their recommendations. These can include measures of adherence or outcome. Performance measures that may be useful in the prevention of infection associated with combat-related injury include:

- Use of a recommended antimicrobial versus other antimicrobial or combination of antimicrobials for postinjury therapy.
- Time from injury to delivery of postinjury antimicrobials.
- Change in rates of colonization with MDR bacteria at admission to tertiary care medical facilities outside the combat zone.
- Change in rates of infection with MDR bacteria during care at tertiary care medical facilities outside the combat zone.

Admission screening for colonization with MDR has been established at the major US military medical centers receiving wounded from the combat zone. This screening was standardized in 2008 to allow comparison among facilities.[285] Monitoring the change in rates of colonization of combat-injured personnel at admission will in part allow assessment of the benefit of these guidelines.

In addition, the Joint Theater Trauma System, which has a performance improvement project which gathers data to inform medical leaders about wounding patterns, effectiveness of interventions, and emerging trends, has recently added an infectious disease module. The Joint Theater Trauma Registry has recently added an infectious disease module which will allow assessment of the effectiveness of the recommendations in this guideline and provide data for future refinements/updates.

The Department of Defense-Veterans Administration Trauma Infectious Disease Outcomes Study is an observational cohort of infectious disease outcomes after deployment-related traumatic injury in active duty personnel or Department of Defense beneficiary from their initial arrival from the combat theater to posthospitalization follow-up. Trauma history and infectious disease-specific inpatient care information is captured through the Joint Theater Trauma Registry. Assessment of postinjury antimicrobial prescribing practices has already been implemented to monitor adoption of the current guidelines. Outcomes analysis of infectious complications in addition to infection rates secondary to MDR bacteria will also be accomplished through this study.

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Prevention of Infections Associated With Combat-Related Thoracic and Abdominal Cavity Injuries

AQ: 1

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Abstract: Trauma-associated injuries of the thorax and abdomen account for the majority of combat trauma-associated deaths, and infectious complications are common in those who survive the initial injury. This review focuses on the initial surgical and medical management of torso injuries intended to diminish the occurrence of infection. The evidence for recommendations is drawn from published military and civilian data in case reports, clinical trials, meta-analyses, and previously published guidelines, in the interval since publication of the 2008 guidelines. The emphasis of these recommendations is on actions that can be taken in the forward-deployed setting within hours to days of injury. This evidence-based medicine review was produced to support the *Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update* contained in this supplement of *Journal of Trauma*.

Key Words: Combat, Trauma, Thorax, Abdomen, Infection, Prevention.

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The ominous nature of penetrating thoracic or abdominal wounds was recognized by ancient physicians, who observed that even those who survived the initial injuries were likely to succumb if infection ensued. The higher velocity penetrating thoracoabdominal injuries of modern warfare were initially distinguished by such high mortality rates that US Civil War patients with these injuries were often treated expectantly.[1] Even now, combat injuries to the chest and abdomen, although not as frequent as extremity injuries, are more commonly serious or fatal and more frequently associated with infectious complications than other sites of injury.[2–4]

Among 486 autopsies from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF, in Afghanistan), 83% of deaths were from penetrating injury and 50% of deaths were attributed to truncal hemorrhage (includes thorax and abdomen), making it the leading cause of death.[4] Another study looking at the cause of death among 82 US Special Operations Forces in Iraq revealed that truncal hemorrhage accounted for 47% of the mortalities.[5]

Management of thoracoabdominal wounds has evolved along with the development of more lethal weaponry and more effective protective equipment. The use of body armor in OIF/OEF, and a shift from bullet wounds to blast injuries from improvised explosive devices (IEDs) have presented new challenges for these treating potentially massive injuries.[6–8] We focus on initial management of chest and abdominal wounds to prevent infection. The data reviewed places emphasis on combat-related studies and case series, especially those from 2007 through 2010 (since the last review).[9]

METHODS

AQ: 2

A Medline search using PubMed from the US National Library of Medicine National Institutes of Health was performed using the key words "abdominal," "thoracic," "military," "combat," "infection," "prevention," "empyema," "hemothorax," "thoracostomy," "irrigation," "antimicrobial," "culture," "bacterial," "wound infection," "splenectomy," "immunization," "sepsis," "meningococcus," "pneumococcus," and "hemophilus" with an emphasis on June 2007 through January 1, 2011. We also crossed referenced published bibliographies for additional manuscripts. In addition, we analyzed ongoing research projects with data published in abstract form or preliminary draft manuscripts for inclusion in the guidelines.

THORACIC WOUNDS

Chest trauma is the second most common cause of traumatic death in the United States (after head trauma) and accounts for approximately 20% of these deaths.[10] Penetrating chest wounds, especially when associated with abdominal injury or esophageal perforation, have been associated with high mortality rates.[2] Borden,[11] in a presentation to the Association of Military Surgeons in 1900, discussed the increased mortality associated with penetrating thoracic wounds caused by high velocity and large caliber rounds

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versus those associated with low velocity and small caliber rounds. A similar comparison exists today between the generally low velocity stab and small caliber gunshot wounds to the chest described in the civilian sector versus the large caliber, high velocity penetrating injuries, and high energy blast injuries experienced in the current military experience. Propper et al.,[12] in a recent review of the data from the US Joint Theater Trauma Registry (JTTR) from Iraq and Afghanistan, revealed that among 33,755 casualties, thoracic injuries were experienced by only 4.9%. This is in contrast to data from Vietnam where 20% of hospital admissions were for thoracic wounds.[13] The OIF/OEF chest wounds are notable for fewer penetrating truncal injuries (40%) and more blast injuries (46%); a contrast from the predominance of bullet and shrapnel penetrating injuries in previous conflicts. The Spanish Army Hospital in Afghanistan noted that 17% of ICU admissions were due to thoracic injuries and that thoracic blast injuries were more likely to require ICU admission than wounds from firearms.[14] In OIF/OEF, lung contusion is the most prevalent thoracic injury, experienced in 32% of cases, with traumatic pneumo- or hemothorax experienced in 19% (Table 1).[12]

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The increase in blast injuries to the chest may explain the significant increase in the mortality associated with thoracic wounds in OIF/OEF (12%) versus Vietnam (3%).[12,13] Although body armor prevents most penetrating thoracic injuries, it does not diminish high energy blast effects. A British study of IED injuries among UK and US forces in Iraq and Afghanistan determined that only 10% of those injured by IEDs suffered torso wounds and a US study found that 80% of thoracic wounds were caused by explosions.[7,15,16]

TABLE 1. Breakdown of 1,660 Thoracic Injuries Sustained in OIF/OEF (Modified From *Ann Thorac Surg.* 2010;89:1032–1036)

Diagnosis	N (%)
Lung contusion	518 (31.8)
Traumatic pneumothorax/hemithorax	316 (19.4)
Rib fracture	215 (13.2)
Diaphragm injury	123 (7.5)
Open chest wound	110 (6.7)
Lung laceration	91 (5.6)
Innominate/subclavian injury	43 (2.6)
Other open thoracic injury	36 (2.2)
Other closed thoracic injury	22 (1.3)
Sternum fracture	22 (1.3)
Intercostal/mammary artery injury	22 (1.3)
Heart laceration	21 (1.3)
Larynx/trachea fracture	19 (1.2)
Esophageal injury	17 (1.0)
Open tracheal wound	12 (0.7)
Flail chest	12 (0.7)
Thoracic vein injury	7 (0.4)
Pharyngeal wound	6 (0.4)
Pulmonary vein injury	4 (0.2)
Vena cava injury	4 (0.2)

Regardless of the etiology of the penetrating wound to the chest, the need to evacuate debris and clot and close open wounds to prevent infection has been a standard practice for over a century, as development of infection was frequently associated with death if a patient survived the initial trauma.[17] The role for postinjury antimicrobials and the duration of their administration in the management of thoracic injuries and thoracostomy has been controversial throughout the antimicrobial era.[18,19]

Prevention of Infection in Traumatic Thoracic Wounds

Famous Second World War surgeon Major Thomas Burford, in his treatise on posttraumatic empyema opines, "Of all the tragic sequelae of war, few are more distressing than the problems of those whose injuries result in chronic intrapleural sepsis. These unfortunates are inevitably found in large numbers through the postbellum years either doggedly submitting to one major operative procedure after another, or resignedly suppurating through a shortened life-span of chronic invalidism."[20]

Prompt surgical intervention with debridement and evacuation of hemothorax combined with appropriate use of antimicrobials has significantly reduced the morbidity and mortality associated with combat-associated chest trauma from 63% in the Civil War to less than 5% in the last 50 years.[1,12,21]

Tube thoracostomy, video-assisted thorascopic surgery, or thoracotomy is used to reexpand the lung and drain fluid, debris, and blood from the chest. Blood accumulating in the pleural space, particularly if a large volume, will form a clot. Retained clot (residual hemothorax), if not evacuated, will organize and adhere to the lung and pleura. Retained hemothorax is difficult to remove, forms a nidus for infection and fibrosis, and is the predominant risk factor for infection after thoracic trauma.[22,23]The incidence of empyema in chest wounds has, in most studies, been higher in combat-related injuries than in civilian, peacetime injuries.[18]

Empyema, although more common after penetrating chest injuries than after blunt chest trauma, may occur with either mechanism of injury (or even in the absence of chest trauma). Some etiologies of empyema are summarized in Table 2.[18,24] The incidence of posttraumatic empyema after chest injuries varies from 2% to 25%, but in most recent series is less than 5%.[19,24–28] Mandal et al.[27] reviewed 5,474 trauma patients (4,584 with penetrating trauma and 890 with

T2

TABLE 2. Etiologies of Empyema After Chest Trauma[18,24]

Direct infection from the penetrating injury and debris in the pleural cavity
Iatrogenic introduction during the performance of thoracostomy
Diaphragmatic disruption and intra-abdominal wound contamination
Secondary infection of undrained or partially drained hemothoraces
Hematogenous spread from infection outside the chest
Development of a parapneumonic empyema from a posttraumatic pneumonia
Pulmonary contusion

blunt injuries) who required tube thoracostomy in Los Angeles over a 24-year period. Among the patients with isolated thoracic trauma, only 1.6% developed posttraumatic empyema and the only significant associated risk factor was retained hemothorax. In a retrospective study of 71 patients who developed empyema (of 2,261 trauma patients with thoracostomy), factors associated with increased risk of empyema included longer duration of thoracostomy, length of ICU stay, presence of contusion, and need for exploratory laparotomy. Retained hemothorax was associated with an odds ratio of 5.5 and was the greatest risk factor observed for development of empyema.[25] Approximately all studies have demonstrated that penetrating chest wounds are more frequently associated with empyema than blunt trauma.

A trauma patient with a pneumothorax or hemothorax requiring tube thoracostomy should have the procedure performed as soon as it is possible to safely do so. In combat settings, medics and corpsmen responding to an injured troop in the field may not have adequate training to perform tube thoracostomy. In the civilian setting, mobile trauma teams have increasingly included a provider with tube thoracostomy training, so it can be performed in the field if the patient is in extremis. In the noncombat literature, there has been considerable controversy regarding the setting and appropriate level of training for a provider to perform tube thoracostomy. Some studies have demonstrated increased complication rates, especially residual hemothorax or empyema, when chest tubes have been placed by providers other than surgeons.[29] Other studies have concluded, there is little difference in outcome with different providers.[30,31] Regardless of who performs tube thoracostomy, it is important to reassess for adequacy of drainage of hemothorax (and possible migration of the tube during transport of the patient) as early evacuation of residual clot is important to diminish risk of developing an empyema.[32]

Postinjury Antimicrobials

Rationale

The role of postinjury antimicrobials in chest trauma to prevent empyema and, to a lesser degree, pneumonia, has remained controversial for decades.[33] As noted previously, in most series the incidence of posttraumatic thoracic infection is low, making significant differences in infection rates between groups administered or not administered postinjury antimicrobials difficult to determine. Individual randomized controlled trials have been underpowered and meta-analyses have reached contradictory conclusions. Overall, eight studies favored the recommendation for postinjury antimicrobials,[26,34–38] contrasting with three not supporting routine use.[27,33,39,40] The meta-analyses have struggled with which of the numerous studies to include due to differences in the choice, dosage, and duration of antimicrobials used and consideration of pneumonia versus empyema (should empyema be considered separately from concomitant pneumonia).[19,33,41] Most authors have concluded that another randomized controlled trial is required to definitively address the issue, but approximately 2,500 patients would be needed to power such study properly.

In 2000, the Eastern Association for the Surgery of Trauma (EAST) guidelines concluded, there were insufficient data to support the use of prophylactic antibiotics for tube thoracostomy as the standard of care or to suggest they reduce the incidence of empyema, but did recommend prophylactic use of a first-generation cephalosporin to reduce the incidence of pneumonia, recommendations that only increased the controversy.[18,42] Guidelines from the British Thoracic Society in 2010 recommended consideration of prophylactic antibiotics in trauma, especially with penetrating chest injuries.[43]

Antibiotic selection

Recommendations for postinjury antimicrobial therapy are to prevent early infection and sepsis, not for the empirical treatment of established infections after chest trauma. The majority of wounds, especially thoracic wounds, are not contaminated with resistant organisms at the time of injury.[44] Most of the organisms isolated have been staphylococcal and streptococcal species.[24,45,46] Although a wide range of organisms have been reported in association with posttraumatic empyema and reports of multidrug-resistant (MDR) gram-negative bacteria and methicillin-resistant *Staphylococcus aureus* have appeared after combat injury, these have been primarily isolated from patients days or weeks after their injury with a sufficient interval of time for acquisition of resistant bacteria from the healthcare system.[28] Empiric antimicrobial coverage for these resistant organisms at the time of injury is therefore not recommended.

Randomized control trials have used a wide range of antimicrobial agents, including amoxicillin, doxycycline, clindamycin, and cephalosporins, at various dosing interval and duration. Although there is not clear evidence that one regimen is preferable to another, cefazolin has been the antimicrobial most frequently studied. Even in a study that markedly under-dosed cefazolin (500 mg intravenously every 8 hours), there was a significant decrease in early pneumonia but not empyema.[26] Cefazolin is also inexpensive, widely available, and is recommended in our guidelines for postinjury treatment for injuries at other sites.[47] Use of a higher dose, 2 g every 6 hours to 8 hours is emphasized, especially in patients who have prolonged surgical procedures and/or significant blood loss. Dosage for children less than 40 kg should be 20 mg/kg to 30 mg/kg IV every 6 hours to 8 hours (up to a maximum of 100 mg/kg/d). Chest wounds with evidence of esophageal perforation have a much wider variety of bacterial contamination that should prompt use of the same antibiotic recommendations as in abdominal wounds (see below).

Duration

Duration of postinjury antimicrobial coverage for surgery, regardless of the site, has remained controversial but prolonged courses, even after severe trauma, are increasingly recognized for their association with MDR organisms if infection develops.[48] Postinjury antimicrobial regimens in chest trauma have ranged from a single dose before tube thoracostomy to continuation of antibiotics for the duration of chest tube drainage. Recommendations from the National Surgical Infection Project for patients undergoing routine

preoperative thoracic surgical procedures (not related to trauma) recommend 24 hours of therapy with cefazolin or cefuroxime.[49] There are no randomized controlled trials assessing the duration of antimicrobials specifically for thoracic trauma without tube thoracostomy. Velmahos et al. retrospectively assessed 250 severely traumatized patients, including 74% who underwent a thoracic or abdominal surgical procedure. Patients received either 1 day of a single antimicrobial or one or more antimicrobials for more than 24 hours, typically 3 days to 5 days. The only significant difference in outcome between the groups was the increased incidence of antimicrobial-resistant bacteria cultured from 50% of those with longer regimens versus 35% of those on short-term regimens.[46] There are no data in trauma to suggest any added advantage to longer durations of postinjury antimicrobials beyond 1 day and a single dose preprocedure is often advocated.

Redosing of antimicrobials in prolonged surgery or in cases of extensive blood loss

To remain effective in preventing infection, a postinjury antimicrobial should maintain a concentration sufficient to inhibit or kill bacteria. Massive blood loss can be associated with thoracoabdominal injuries due to disruption of the great vessels and/or lengthy surgical repair of extensive injuries. A 1,500 mL to 2,000 mL blood loss (or more) accounts for 30% to 40% of a patient's blood volume and replacement of that volume of blood suggests that serum antimicrobial concentrations may be diminished. Many surgeons have addressed this by empirically decreasing the dosing interval in cases requiring a significant volume of blood products. Evidence to support this practice has been somewhat conflicting. Cefazolin drug levels have been the most studied. As cefazolin does not enter red blood cells, it is the loss of plasma, not total blood volume that is responsible for any decrement in the plasma concentration. A number of the studies that demonstrated little change in serum levels of cefazolin were associated with smaller volume blood losses (1,200 mL or less).[50–52] Meter et al.[53] prospectively studied 18 patients undergoing hip surgery and assayed cefazolin levels 48 hours before operation and during surgery. Even though the average blood loss was 1,137 mL, there was no clinically significant decrement in cefazolin levels. Furthermore, Meter et al. extrapolated their pharmacokinetic data to calculate that even with a blood loss of 5,000 mL, there would be adequate serum levels of cefazolin. Swoboda et al.[54] prospectively studied 11 patients undergoing spinal surgery and performed pharmacokinetic measurements of serum and tissue concentrations of cefazolin and gentamicin throughout the procedure and concluded that additional doses of cefazolin should be administered in cases with more than 1,500 mL of blood loss or surgery longer than 3 hours.

The pharmacokinetics for most antimicrobials have not been adequately assessed in trauma patients and the data are insufficient to support specific recommendations for altering dosing regimens for other agents.[55]

The 2000 EAST guidelines for postinjury antimicrobials in penetrating abdominal trauma concluded that there were insufficient data for evidence-based recommendations but advised that in cases of massive hemorrhage, antibiotic dose should be doubled or tripled and repeated after every tenth unit of blood product transfusion.[56]

Although data are conflicting, it appears that hemorrhage of more than 1,500 mL and development of shock may be associated with altered pharmacokinetics of antimicrobials and potentially inadequate serum concentrations. Our recommendation is for redosing of antimicrobials after large volume blood product (1,500–2,000 mL of blood loss) resuscitation has been completed, regardless of when the last dose of antimicrobial was administered.

Our review of the literature does not support change to the recommendations for thoracic trauma postinjury antimicrobials made in the 2008 guidelines.[47] Although the subject remains controversial, the majority of studies have demonstrated a reduction in both empyema and pneumonia in patients administered antimicrobials postthoracic trauma. The administration of a single dose of cefazolin (2 g IV) before tube thoracostomy or thoracotomy and, if desired, continued (every 6–8 hours) for no more than 24 hours after the procedure, may reduce infectious complications without significant selective pressure on colonizing bacteria yielding antimicrobial resistance.

ABDOMINAL WOUNDS

In 1898, Cousins,[57] a British surgeon, described resuscitation of a patient after an abdominal gunshot wound with "subcutaneous strychnine and brandy." Surprisingly, the patient "rallied" with this medical intervention and went on to laparotomy, debridement, irrigation, and a successful repair of a gastric perforation, to ultimately survive. Although preoperative management has advanced considerably in the last century, many of the surgical principles remain current.

In the US Civil War, penetrating abdominal injuries were associated with death in 87% of cases; poor outcomes were so uniform that surgical intervention was uncommon. As general anesthesia with ether became widely available, surgeons could perform longer, more intricate procedures, and by the close of the 19th century, early surgical intervention for thoracic and abdominal injuries was becoming accepted as potentially lifesaving. Surgeons faced a massive number of injuries in World War I and the British Army's review of data from penetrating combat wounds to the abdomen demonstrated that early surgical intervention was associated with approximately 50% survival and, by 1916, mandated early surgical exploration after penetrating abdominal injury during the remainder of the war.[58] The Belgian surgeon Depage[59] noted that rapid access to surgical intervention was critical in abdominal wounds and that moving "advanced dressing stations" to within 2 km to 3 km of the front, along with adequate debridement and irrigation decreased mortality from 65% to 45%. Even as antibiotics became available in the 1940s the importance of prompt irrigation, debridement, and repair to prevent development of infection have remained paramount.[60–63]

During the First World War operating on patients in shock was associated with worse outcomes and postponement of surgery to treat shock was advocated by some surgeons.[61]

As the concept of delay of definitive repair in patients evolved in the 1980s and 1990s to "Damage Control" surgery, the appropriate timing for closure of the abdomen and selection of suitable prophylactic antimicrobials of narrow versus broad spectrum antibiotics have remained areas of discussion.[64] Although severe abdominal trauma may be associated with multiple intestinal perforations, injuries not causing intestinal perforation have a much lower incidence of infectious complications.[65]

Abdominal trauma in the wars in Iraq and Afghanistan occurred in similar proportions to those seen in the Second World War, Korea, and Vietnam.[2,13,16] In OEF/OIF, abdominal wounds constituted 9.4% of 6,609 wounds recorded by the US JTTR. A total of 81% of abdominal injuries were caused by explosions, 17% by gunshots, and 2% by motor vehicle collisions.[16] Casualties earlier in the war were more likely to have suffered gunshot wound than blast abdominal injuries.[66] Hospital data regarding injuries are skewed by inclusion of only those patients that survive to admission. Because abdominal injuries, like thoracic injuries, may be associated with significant hemorrhage that cannot easily be halted by compression (or having a tourniquet applied) in a tactical setting, many personnel with abdominal injuries die around the time of injury. Among 486 autopsies from OEF/OIF, 83% of deaths were from penetrating injury and 50% of deaths were attributed to truncal hemorrhage (includes thorax and abdomen), making it the leading cause of death.[4]

The incidence of postinjury infection in penetrating abdominal injury reported in the literature ranges from 4% to 31%.[67–70] A study of 211 injured patients cared for on the USNS Comfort during the first months of the Iraq War found 30% of abdominal injuries were infected, yielding an odds ratio of 2.7 for an abdominal injury to develop an infection (this series was primarily civilian Iraqis, not US troops).[28] In civilian studies, Nichols et al.,[65] in a study of 145 patients with abdominal trauma and gastrointestinal perforation, identified increased age, injury to the left colon requiring colostomy, large numbers of intraoperative blood products and a larger number of injured organs as factors associated with an increased risk of postoperative infections. Croce et al.[71] and later O'Neill et al.[69] found a significantly increased number of infections in patients with concomitant gastric and colonic perforation over those with isolated colonic perforation. More recently, Salim et al.[70] analyzed outcomes of 178 cases of penetrating stomach and small bowel injuries and reported 50% of combined stomach and colon perforations developed postoperative infections while only 16% of isolated gastric injuries developed an infection.

The organisms responsible for infections after penetrating abdominal trauma have been well characterized in numerous studies and are most commonly *Escherichia coli* and other *Enterobacteriaceae*, *Streptococci* (including *Enterococcus* spp.) and *Bacteroides* spp. Colonic perforations are more likely to be associated with *E. coli* and *Bacteroides* spp., while *Enterobacter cloacae* and *Klebsiella* spp. are seen more commonly in gastric and small bowel injuries. *Candida* spp. have been reported in 20% of infections in a recent study.[67,69,70] Although there has been understandable concern about the prevalence of MDR gram-negative bacteria, including extended-spectrum β-lactamase (ESBL)-producing *E. coli, Klebsiella* spp., and *Enterobacter* spp. and MDR *Acinetobacter baumannii* in abdominal infections, it appears that most of these resistant organisms have been acquired from the healthcare system and not at the time of injury.[28,44] They are, therefore, not targets of antimicrobial prophylaxis.

Prevention of Infection in Traumatic Abdominal Wounds

Postinjury Antimicrobials

Rationale

Penetrating abdominal injury is so frequently associated with bacterial contamination that postinjury antimicrobials have become the standard of care.[72] Unlike thoracic trauma, routine administration of postinjury antimicrobials in penetrating injuries to the abdomen had come into practice during the Korean War and was well established by the 1970s. In 1972, Fullen et al.[73] conducted a retrospective study of 295 patients who underwent laparotomy after penetrating abdominal injury first demonstrated that antimicrobials administered preoperatively were associated with significantly lower rates of secondary infection. They observed fewer infectious complications in those who received antimicrobials preoperatively (7%) than when given intraoperatively (33%) or postoperatively (30%). Subsequent studies by Thadepalli et al.[74] compared presurgical administration of kanamycin plus cephalothin with the expanded anaerobic coverage provided by kanamycin plus clindamycin and saw significantly fewer postoperative infections (27% vs. 10%) in the clindamycin group. The kanamycin plus cephalothin group experienced anaerobic infections in 21% versus only 2% in the patients treated with kanamycin/clindamycin. These high quality studies supported recommendations and guidelines decades later.[56,75] The use of postinjury antimicrobials in abdominal trauma has become the standard of care and subsequent studies over the next 40 years have not been placebo-controlled, but comparisons between different regimens and have included combinations of nearly every antimicrobial class, dosage, and duration.

Despite near universal acceptance and guideline recommendations, Brand et al.,[76] in a 2009 Cochrane Review determined that none of more than 500 references reviewed constituted a randomized controlled trial that fulfilled their strict inclusion criteria. They therefore concluded that recommendations in guidelines for postinjury antimicrobials in abdominal trauma are based on expert opinion rather than firm evidence from clinical trials. We disagree with their conclusions. It is our opinion that there are adequate trials to support our recommendations.

There has also been considerable controversy about the choice of antimicrobials recommended for postinjury administration (and for treatment of established infections) after perforating abdominal injury. The Surgical Infection Society and the Infectious Diseases Society of America Guidelines Committee, in both 2002 and 2010, in making recommendations for treatment of established intra-abdominal infections concluded that there were insufficient data to recommend a

single regimen as superior to others based on efficacy.[75,77] A similar conclusion can be drawn for postinjury regimens. Many of the trials comparing postinjury regimens were not designed to detect therapeutic superiority and were underpowered to even detect a significant difference between the treatment groups. There have been scores of different antimicrobial combinations compared, many of which were reviewed in forming the EAST Practice Management Guidelines for postinjury antimicrobial use in penetrating abdominal trauma in 2000.[56,78–82]

Although many different antimicrobials, either alone or in combination, can be considered for postinjury administration in abdominal penetrating trauma, there are some factors that should be considered in the determination of which drugs to use. An ideal regimen provides antimicrobial coverage for enteric gram-negative bacteria, primarily the *Enterobacteriaceae*, *Streptococci*, and anaerobes, predominately *Bacteroides* spp. Metronidazole remains overall a highly effective anaerobic antimicrobial. A number of recent studies suggest that clindamycin is inferior to metronidazole, carbapenems, and moxifloxacin for treating anaerobic infections due to its poor coverage of *Bacteroides* spp., the primary cause of anaerobic infection in penetrating abdominal wounds, and other clinically relevant anaerobes such as *Prevotella* spp.[83–88] In the 10 years since the EAST Guidelines were published, there have been additional studies performed and a trend toward the recommendation of a single dose of a single agent for prophylaxis. Both ertapenem and moxifloxacin have been shown to have at least comparable efficacy with established single- and dual-drug regimens in prophylaxis for elective (nontrauma) surgery and treating established intra-abdominal infections.[89,90] Ertapenem was superior to cefotetan in a randomized double blind trial for elective colorectal surgery, a difference likely due to the modest anaerobic activity of cefotetan.[91] Assumptions that drugs with demonstrated efficacy in elective abdominal procedures will perform equally well in severe trauma cases must be made cautiously. Serum levels and pharmacokinetics of ertapenem and moxifloxacin have been performed in healthy adults, not in critically injured patients who may have experienced massive hemorrhage and shock. There are no data on the pharmacokinetics of ertapenem in the trauma patient and relatively little experience with its use in trauma patients. The pharmacokinetics of ertapenem in eight critically ill patients with sepsis demonstrated wide variability in comparison with healthy volunteers with suboptimal serum drug concentrations observed in some patients. The authors questioned whether it was even appropriate to use ertapenem in septic patients.[92] Moxifloxacin has been more thoroughly evaluated than ertapenem. For example, in one study of 10 patients with peritonitis, serum, and peritoneal concentrations of moxifloxacin were measured. The peritoneal fluid achieved higher concentrations than plasma and exceeded the minimal inhibitory concentration for the most common pathogens.[87] Moxifloxacin was also studied in two comparison trials in the treatment of complicated intra-abdominal infections and found to be comparable with ceftriaxone plus metronidazole.[77,90] There are no data for the use of moxifloxacin in postinjury for abdominal wounds.

A combination of cefazolin and metronidazole is recommended in the updated guidelines.[47] This selection is based on evidence of the efficacy of these agents, years of experience with their use in a variety of surgical scenarios, and because they are used for postinjury treatment for other injury types. Use of this combination allows a more limited number of agents to be stocked in a forward deployed setting, especially in this setting where there is no evidence that any alternative regimens are more efficacious. Either ertapenem or moxifloxacin are acceptable alternative agents for postinjury antimicrobial therapy. These agents provide simple regimens that may be preferred by some surgeons, or in some situations. Neither ertapenem nor moxifloxacin have good data supporting their use in trauma patients. Furthermore, limiting use of quinolones, carbapenems, and expanded-spectrum cephalosporins should decrease the selective pressure on enteric bacteria and development of resistance should a postoperative infection develop.

Although there has been understandable concern about the prevalence of MDR gram-negative bacteria, including extended-spectrum β-lactamase (ESBL)-producing *E.coli, Klebsiella* spp., and *Enterobacter* spp., and MDR *Acinetobacter baumannii* in established abdominal infections, it appears that most of these resistant organisms have been acquired by transmission from the healthcare system,[28] and not at the time of injury.[27] Empiric coverage for these resistant organisms in postinjury regimens is not recommended.

Redosing of antimicrobials (see discussion in thoracic section) should be considered after large volume resuscitation with blood products (1500–2000 mL) has been completed, regardless of when the previous dose was administered.

Antibiotic impregnated beads, cement, and sponges have been used by surgeons to prevent infections in a variety of capacities. A gentamicin collagen sponge has been approved for surgical implantation in many countries and has been used in over a million patients. Bennett-Guerrero et al.[93] randomly assigned 602 patients undergoing colorectal surgery to either have placement of two gentamicin-collagen sponges or no sponges and paradoxically observed a significant increased incidence in superficial surgical-site infections (30% in the sponge group and 21% in controls). Antibiotic sponges should not be considered as part of a prophylactic regimen in abdominal trauma surgery. Topical antimicrobials have also been widely studied and appear of no added value if systemic postinjury antimicrobials are provided.[94]

Duration of postinjury antimicrobials

The pharmacologic goal of antimicrobials in abdominal trauma is to ensure a sufficient concentration of a suitable agent is present in the peritoneal cavity during the vulnerable period before the establishment of infection. At laparotomy the perforation is closed, the field is irrigated to reduce peritoneal contamination and no further antimicrobials should be required.[56] Straightforward as this goal is, the optimal duration of postinjury antimicrobials in penetrating abdomi-

nal trauma has remained controversial. A number of studies have demonstrated that longer courses (greater than 24 hours) offer no advantage over shorter (less than 1 day) regimens.[95–98] Dellinger et al.[98] randomized 116 patients with confirmed penetrating injuries of the bowel to either 12 hours or 5 days of postinjury antimicrobials, 24% developed a postoperative infection, but there was no difference in the incidence of infection between the two groups. In a larger prospective study of 515 patients, Fabian et al.[96] randomized patients to 1 day or 5 days of antimicrobial coverage and again found no difference in the incidence of infection between the two groups even in those with the more severe colonic perforations. The EAST guidelines that were published in 2000 recommend that the chosen postinjury antimicrobial dose be administered once preoperatively and, if there is no evidence of gastric or bowel perforation at laparotomy, limit administration to a single dose. If gastric or bowel perforation is identified, then antimicrobials are continued for no more than 24 hours.[56] Despite recommendations for short-course regimens, there is reluctance to adhere to these recommendations, especially when there has been colonic perforation. Delgado et al.[68] observed that postinjury guidelines for penetrating abdominal injury were exceeded in 78% of cases and even observed a trend toward increased infections in those patients who had received prolonged antimicrobials.

SURGICAL MANAGEMENT

Recommendations

For patients with abdominal and thoracic injuries, the skin should not be closed if there is a colon injury or extensive devitalized tissue due to extensive contamination, shock, or residual injured tissue at the incision site. Similarly, skin incisions should not be closed even if possible in the presence of massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high velocity injury, or extensive local tissue damage.

Early primary repair of complex or destructive colonic injuries should not be performed especially if associated with massive blood transfusion, ongoing hypotension, hypoxia, reperfusion injury, multiple other injuries, high velocity injury, or extensive local tissue damage.

If the abdomen is left open, the possibility of partial or complete closure should be considered at each subsequent laparotomy. Scheduled laparotomies should be performed in patients managed with an open abdomen technique at 24-hour to 48-hour intervals.

Since the original recommendations, several additional studies in combat casualties have been published which serve only to further confirm the original guidelines.[9,99,100] One study by Duncan et al.[101] documented the outcome of 23 combat casualties with colorectal injuries. Management of these injuries resulted in 30% undergoing primary repair, 13% undergoing resection and anastomosis, and 57% undergoing diversion with colostomy. Four of these patients were initially managed operatively via a "damage control" laparotomy, and in each case, they were ultimately managed with colostomy as definitive treatment for their colon or rectal injury. Of note, 30% of patients treated with either primary repair or resection and anastomosis went on to develop a leak and required diversion, compared with none in the diversion group. The authors concluded that based on injury severity, the complex nature of triage and medical evacuation and the multiple levels of care involved for injured military personnel, temporary stoma usage in patients with penetrating colorectal injuries should play a greater role in the military population than in the civilian environment. In a slightly larger study done by Vertrees et al.,[102] the authors retrospectively evaluated 65 patients with major colon injuries, 92% of whom had penetrating injuries. The authors documented a primary repair rate of 57% and a 43% diversion rate. Failure of repair occurred in 16% and was more likely in those with concomitant pancreatic, gastric, splenic, diaphragmatic, and renal injuries. In a subset of patients who underwent colon injury damage control (n = 27), delayed anastomoses were performed in 10 patients and 17 patients were treated with diversion. In the damage control subset, 50% (n = 5) of the patients undergoing delayed anastomoses went on to develop a leak and ultimately required a second diversionary procedure. The authors concluded that primary repair of war-related colon injuries could be performed safely in a selected patient population in the absence of concomitant organ injury, as was evident in the damage control group.

Finally, Cho et al.[103] retrospectively reviewed 133 patients who sustained colonic injuries from penetrating (71%), blunt (5%), and blast (23%) mechanisms. Authors divided the cohort into three groups: initial primary repair (32%), initial diversion (44%), and initial damage control (23%). All three groups had similar colon-related complication rates (14%, 15%, and 20%), and there were no identified risk factors on multivariate logistic regression analysis for colon-related complications. On discharge from the institution, a total of 62% of the study cohort had undergone a diversion and 38% had undergone either a primary or a delayed repair. The authors concluded, similar to other articles, that in a combat setting, primary repair is feasible with acceptable complication rates in selected cases.

For severe blunt and penetrating abdominal injuries, damage control principles are indicated and the resulting open abdomen requires careful management to prevent infection and promote healing. Several recent studies have advocated the use of negative pressure wound therapy (NPWT, also called vacuum-assisted closure devices) in the management of these patients.[104–106] Miller et al.[107] studied the use of NPWT in a prospective, single center, comparative protocol. The authors concluded that the rate of successful primary fascial closure (88%) and the time to fascial closure were both significantly improved with the use of NPWT compared with historical controls. No enterocutaneous fistulas were reported. However, patients required frequent trips to the operating room for NPWT changes (every 24–72 hours until fascial closure). In a similar study, Suliburk et al.[108] documented a fascial closure rate of 86% in patients with open abdomens treated with NPWT. No enterocutaneous fistulas were reported and time to fascial closure was 7.0 days ± 1 days. Recent studies in combat casualties undergoing aeromedical evacuation using NPWT documented their safe use

during flights and a similar benefit in wound closure.[109,110] The use of these devices appears to be both safe and effective in patients with open abdomens.

IMMUNIZATION IN THE EVENT OF SPLENECTOMY

Recommendation

Immunize with pneumococcal, *Hemophilus influenza* type b (Hib), and meningococcal vaccines as soon as the patient is clinically stable and preferably within 2 weeks of splenectomy. A single booster dose of pneumococcal vaccine should be administered 5 years later. A booster dose of meningococcal vaccine should be administered 2 months after the initial dose and every 5 years thereafter.

Rationale

Overwhelming postsplenectomy infection (OPSI) is a rare and potentially fatal condition that can develop weeks to years after splenectomy. Although these infections have been associated with many different bacteria, the encapsulated organisms, especially *Streptococcus pneumoniae* (50–90% of cases),[111] *Hemophilus influenzae* type b (Hib), and *Neisseria meningitidis* are the most likely to cause severe, invasive disease in individuals with asplenia. Patients suffering from OPSI may progress from good health to death in only 12 hours to 18 hours.[112] The greatest risk of OPSI is in children, especially those younger than 2 years of age, but fulminant sepsis may occur at any time, with OPSI being reported from 24 days to 65 years postsplenectomy.[111] A meta-analysis of literature from 1952 to 1987 of 5,902 patients found an incidence of 4.4% and mortality of 2.2% in children younger than 16 years. In adults, the incidence was 0.9% with a mortality of 0.8%.[113] There is a lower incidence of OPSI in adults who have had a splenectomy posttrauma versus splenectomy for neoplasm or other medical diagnoses. Another extensive review estimated that asplenic patients did not experience a significant increase in the risk of sepsis beyond that in the general population, but that there was a 58-fold increased risk of death among asplenic patients who developed sepsis.[114] Although there are not adequate randomized control studies to yield strong evidence to support immunization against these agents after splenectomy, the practice is currently recommended by the Surgical Infection Society,[115] the Advisory Committee on Immunization Practices (ACIP) of the Centers for Disease Control and Prevention (Table 3),[116] and in the Clinical Practice Guidelines of the Joint Theater Trauma System for patients postsplenectomy.[117] T3

Pneumococcal Immunization

There is evidence that immunization after splenectomy yields antibody titers up to 50% lower than when administered before splenectomy (although there are contradictory studies).[118,119] Furthermore, the timing for immunization after a traumatic splenectomy remains controversial. The randomized clinical trials are only for pneumococcal vaccine and have had some conflicting findings.[120] In a series of studies using the 23-valent pneumococcal polysaccharide vaccine after splenectomy for trauma, Shatz et al.[121,122] found that immunization given at days 1, 7, 14, or 28 after splenectomy were all associated with an immune response. The antibody levels achieved with immunization at days 7 or 14 were significantly lower, probably reflecting the suppression of the immune system immediately after trauma and surgery. Although immunization after splenectomy yields lower functional antibody titers than when administered with an intact spleen, the antibody levels achieved at 14 days postsplenectomy were equivalent to those at 28 days after surgery. Other human studies have failed to demonstrate any significant difference in antibodies in immediate versus delayed immunization.[119] With both polysaccharide and conjugate vaccines available, there remains no strong evidence to use one vaccine over the other. The current recommendations from the ACIP are to administer the 23-valent polysaccharide pneumococcal vaccine in asplenic children and adults. Additionally, asplenic children should be administered the pneumococcal conjugate vaccine on the same schedule as is recommended for children with an intact spleen.[123] Studies in both Britain[111] and the United States[124] have demonstrated that despite recommendations for the use of all three vaccines that immunization often does not occur.

Meningococcal Immunization

Asplenic persons who develop meningococcal infection have mortality rates of 40% to 70%. A study with a meningococcal conjugate vaccine demonstrated that 20% of asplenic persons do not develop adequate serum bactericidal activity after a single dose of vaccine but a second dose 2 months later reduced those with inadequate titers to 7%.[125,126] ACIP meningococcal recommendations for those with asplenia have recently been modified and now recommend a two-dose primary series with the second dose of meningo-

TABLE 3. Recommended Immunizations After Traumatic Splenectomy (ACIP)[116,126]

Vaccine	Primary Series	Repeat Vaccination
23-valent Pneumococcal polysaccharide	When clinically stable, preferably within 2 wk of splenectomy (children <5 yr should receive age appropriate Pneumococcal conjugate vaccine in addition)	Single repeat dose 5 yr later
Polysaccharide protein conjugate *Hemophilus influenzae* b	When clinically stable, preferably within 2 wk of splenectomy	No recommendation for repeat
Quadrivalent Meningococcal conjugate vaccine	When clinically stable, preferably within 2 wk of splenectomy for first dose, second dose 2 mo later	Every 5 yr (at the earliest opportunity if a 1-dose primary series was administered, then every 5 yr)

coccal vaccine administered 2 months after the initial dose and then a booster dose every 5 years. Those who have previously received only a single-dose primary vaccine should receive a second dose at the earliest opportunity and subsequently every 5 years.[126]

Hib Immunization

The data for use of the Hib vaccine after splenectomy is lacking although expert opinion recommends its administration. A single primary dose is recommended, and there are no data regarding subsequent booster doses.

Additional Considerations After Splenectomy

It is important that asplenic patients and their providers are made aware of the increased risk for infections, the recommendations for repeat immunization and the increased risk of sepsis. The role of postsplenectomy antibiotic prophylaxis remains controversial, especially in adults who have been vaccinated. In children, especially younger than 5 years, the incidence of sepsis is so increased that the American Academy of Pediatrics recommends daily antibiotic prophylaxis penicillin be considered, particularly for the first year after splenectomy.[115,127]

RESEARCH GAPS

Trauma is inherently a difficult area in which to perform randomized controlled trials. The lack of adequately powered studies to answer questions such as the optimal antimicrobial regimens for postinjury administration in abdominal trauma will therefore remain controversial. Likewise, the pharmacokinetics of antimicrobials in severe, combat injuries has not been adequately assessed. Generalizing antimicrobial recommendations made for elective thoracoabdominal surgery to the severely traumatized patient may be inaccurate and further research in this population will potentially make recommendations for use of newer agents possible.

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Prevention of Infections Associated With Combat-Related Extremity Injuries

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Abstract: During combat operations, extremities continue to be the most common sites of injury with associated high rates of infectious complications. Overall, ~15% of patients with extremity injuries develop osteomyelitis, and ~17% of those infections relapse or recur. The bacteria infecting these wounds have included multidrug-resistant bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, extended-spectrum β-lactamase-producing *Klebsiella* species and *Escherichia coli*, and methicillin-resistant *Staphylococcus aureus*. The goals of extremity injury care are to prevent infection, promote fracture healing, and restore function. In this review, we use a systematic assessment of military and civilian extremity trauma data to provide evidence-based recommendations for the varying management strategies to care for combat-related extremity injuries to decrease infection rates. We emphasize postinjury antimicrobial therapy, debridement and irrigation, and surgical wound management including addressing ongoing areas of controversy and needed research. In addition, we address adjuvants that are increasingly being examined, including local antimicrobial therapy, flap closure, oxygen therapy, negative pressure wound therapy, and wound effluent characterization. This evidence-based medicine review was produced to support the *Guidelines for the Prevention of Infections Associated With Combat-Related Injuries: 2011 Update* contained in this supplement of *Journal of Trauma*.
Key words: Extremity, Infection, Prevention, Iraq, Afghanistan.

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Historically, the extremities have been the most common sites of injury in combat, and this has remained true during the ongoing wars in Iraq and Afghanistan (Table 1).[1–7] T1 The rate of vascular injuries in modern combat is five times than that reported in previous wars.[8] There are approximately equal numbers of upper and lower extremity injuries; however, lower extremity injuries are more severe, with higher infection rates, especially when associated with a vascular injury (Table 2).[5,9–13] Extremity injuries are associated with T2,AQ:1 major morbidity as evidenced by high complication rates and healthcare utilization. Over a 56-month period, of 5,684 casualties with major limb injuries, 423 (7.4%) underwent major limb amputation, similar to the 8.3% rate during the Vietnam War.[14] A review of 1,333 soldiers revealed that those with extremity injuries had the longest average hospital stay (17.9 days), accounting for $65.2 million total inpatient resource utilization with a projected cost of $170 million disability benefit. Extrapolation of total disability costs for these wars was ~$2 billion.[15]

The goals of extremity injury care are to prevent infection, promote fracture healing, and restore function. Our previous review of combat-related extremity injury infection prevention and management focused on wound debridement and irrigation, initial stabilization, tetanus prophylaxis, systemic antimicrobial therapy, and delayed wound closure.[16] Adjuvant treatments are increasingly being examined to improve outcomes. These include use of local antimicrobial therapy, flap closure, and oxygen therapy, and characterization of wound effluent. In this updated supporting document to the guidelines for prevention of infections associated with combat-related injuries,[17] we use a systematic review of military and civilian extremity trauma data to provide evidence-based recommendations for the varying management strategies. We focus on data primarily from 2007 through 2011 to augment the previous guidelines with an emphasis on antimicrobial therapy, debridement and irrigation, and wound management highlighting ongoing areas of controversy and needed research. We include recommendations as they apply to role (echelon or level) of care: Role 1—self-aid, buddy aid, combat lifesaver, and combat medic/corpsman care at the point-of-injury; physician/physician assistant care but no patient holding capacity. Role 2—72-hour patient holding capacity, basic blood transfusion, and radiography and labo-

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TABLE 1. Review of Combat-Related Extremity Injury Infection Articles Published During the Wars in Iraq and Afghanistan

Reference	Time Frame	Evacuation Time	Antimicrobials	Focus of Study	Subjects	Infectious Information	Bacteria
Helgeson et al.[125]	2001–2006	N/A	N/A	Calcium sulfate carrier for antimicrobials and bone graft substitute	15 patients (17 fractures)	Postoperatively 4 of 18 grafting procedures showed clinical infection, with 13 of 17 having positive intraoperative bacteria 22% rate of osteomyelitis	Intraoperative cultures: 11 *Acinetobacter*, 5 *Staphylococcus*, 2 *Klebsiella*, 2 *Pseudomonas*, 1 each with *Bacteroides*, *Bacillus*, and *Corynebacterium*
Lin et al.[274]	2001–2003	7.9 d	Cefazolin plus gentamicin for dirty wounds	Extremity injuries	52 (15 with traumatic amputations)	No infections in fracture only group; two infections among amputee patients	*Pseudomonas*, MRSA, and *Acinetobacter*
Yun et al.[10]	2003–2006	None listed	Variable include early vancomycin	Orthopedic injuries with osteomyelitis	2,854 admission with 664 admitted to orthopedics; 103 with osteomyelitis 2:1 ratio of lower to upper extremities injuries with osteomyelitis	84 (83%) of these patients did not relapse during a follow-up that ranged from 2 to 36 wk	*A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* isolated during an original episode Gram-positive organisms were more likely during recurrences; *S. aureus* (13% vs. 53%)
Enad and Headrick[2]	2003	N/A	Broad spectrum that included Gram negative coverage	Orthopedic injuries	58 total service member, 30 fractures, and 14 total with battle injury	No perioperative infections	None listed
Hinsley et al.[275]	2003	Rapid, but not detailed	Benzyl penicillin before arrival to hospital	90% Iraqi	39 patients with 50 ballistic fractures (17 upper and 33 lower extremity)	43 evaluable wounds: 13 of 43 became infected, with 5 of 43 deep infections Infection occurred significantly more often with gunshot fractures, wound closed primarily, and intra-articular fractures	None listed
Petersen et al.[74]	2003	4.2 d	Variable	Evacuation to US naval hospital ship	211 patients (179 Iraqi)	44 of 56 extremities injuries developed infection; no fracture data	Most common bacteria—*Acinetobacter*, *E. coli*, *Pseudomonas*, and *Enterobacter* species
Johnson et al.[20]	2003–2006	7.4 d	Variable	Grade III tibial fractures	62 open tibial fractures with 40 grade III (35 with data)	27 with at least 1 organism (deep wound culture) None of the initially recovered Gram negative bacteria; cultured again after being treated for a deep infection or osteomyelitis 5 of 35 patients ultimately required limb amputation with infectious complications cited as the reason in 4	*Acinetobacter* species, *Enterobacter* species, and *P. aeruginosa* were the most commonly recovered bacteria initially Staphylococcal organisms were found in every wound at the time of repeat operation, along with *P. aeruginosa* in three samples

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TABLE 1. Review of Combat-Related Extremity Injury Infection Articles Published During the Wars in Iraq and Afghanistan (continued)

Reference	Time Frame	Evacuation Time	Antimicrobials	Focus of Study	Subjects	Infectious Information	Bacteria
Mack et al.[276]	2003–2007	N/A	N/A	Open periarticular shoulder	44 (33 grade IIIa, 1 grade IIIb, and 10 grade IIIc)	31 of 44 initially cultured of which 22 were positive 1 or more year of follow-up; 5 of 35 became infected (4 IIIa and 1 IIIc)	Of 22 initial cultures, 14 were *A. baumannii*
Possley et al.[206]	2003–2007	N/A	N/A	Safety of external fixation	55 grade III tibia fractures	No cases of pin tract osteomyelitis 8 cases of osteomyelitis at fracture site An additional 22 tibias were clinically diagnosed and treated for osteomyelitis at the fracture site without a positive bone culture Two soft tissue infections	Not listed
Geiger et al.[1]	2003–2005	N/A	Not stated but initially meropenem added until wounds closed	Plastic surgery care	42 patients with lower extremity injury, 20 with upper extremity, and 10 with both	15 of 62 developed acute osteomyelitis, and 1 of 62 developed chronic osteomyelitis	9 *A. baumannii*, 5 *Enterobacter*, 4 coagulase negative *Staphylococcus*, 3 *Enterococcus*, 2 MRSA, 1 *Bacillus*, and 1 *Klebsiella*
Brown et al.[21]	2003–2008	92% within 12 h	Majority penicillin/ floxacilin plus anaerobic coverage	Mangled extremity	84 casualties (85 extremities)	24% developed infection with 6% developed osteomyelitis Fasciotomy, antimicrobials during air evacuation, and *P. aeruginosa* were significantly associated with infectious complications	*S. aureus* were recovered later in casualties' clinical course in contrast to early recovery of *Acinetobacter*
Brown et al.[11]	2003–2008	Less than 6 h 95%	N/A	Vascular injury	37 total (29 with fractures)	20 of 29 limbs with a fracture developed superficial infection and 2 of 8 limbs without a fracture developed a superficial infection 3 of 29 with fracture developed deep infection	None listed
Clasper and Phillips[209]	2003	N/A	N/A	External fixation use	15 external devices (14 patients)	3 fixators developed pin track infections and failed despite antimicrobials	None listed
Mody et al.[19]	2003–2007	N/A	N/A	Damage control orthopedics	58 patients—34 grade IIIa, 9 grade IIIb, and 3 grade IIIc	Fracture site infection 40% and suspected osteomyelitis 17%	Surgical Site infections (23): polymicrobial 44%, Gram negative 65%, and Gram positive 44% Early infections (13)—9 Gram positive and 5 Gram negative Late infection (9)—5 Gram positive and 6 Gram negative

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TABLE 1. Review of Combat-Related Extremity Injury Infection Articles Published During the Wars in Iraq and Afghanistan (continued)

Reference	Time Frame	Evacuation Time	Antimicrobials	Focus of Study	Subjects	Infectious Information	Bacteria
Orr et al.[277]	2003–2008	N/A	N/A	Sural artery flap foot and ankle	10 grade IIIb	3 deep wound infections with osteomyelitis	MDR *Klebsiella* and *Acinetobacter*
Keeling et al.[229]	2004–2007	N/A	Cefazolin plus merorpenem plus vancomycin beads	Ring external fixation	67 grade III tibial shaft fractures with 45 tibia fixations with 36 cases reviewable	Standardized treatment protocol—3 of 38 tibia infected and all treated successfully with debridement and antimicrobials without frame removal	None listed
Leininger et al.[136]	2004–2005	Within 24 h of injury	N/A	Negative pressure wound therapy study	Soft tissue infections—20 upper and 38 lower extremity	No fracture data present No infections described with standard debridement and use of negative pressure wound therapy	None listed
Kumar et al.[13]	2004–2007	N/A	N/A	Upper extremity flaps	23 patients with 26 upper extremity injuries	46% of wound colonized at admission 2 flaps early postop infections with no sequelae	75% *A. baumannii*
Kumar et al.[12]	2004–2007	12 open fractures (75%) nailing within 72 h	N/A	Lower extremity flap reconstruction	43 patients all with grade IIIb and grade IIIc fractures—22% upper extremity, 52% tibia/fibula, and 22% ankle/foot	50% of wounds colonized at admission 8 flaps early infection with no sequelae	57% *A. baumannii*
Keeney et al.[278]	2005–2006	N/A	First-generation cephalosporin plus fluoroquinolone until 48 h after wound closure	Immediate nailing or staged treatment of closed femoral fracture	22 patients (23 femoral fractures) with 16 grade IIIa open fractures	Follow-up 2 mo for 8 and 6 mo for 5 No infections	None listed
Stinner et al.[207]	2001–2008	N/A	N/A	Outcome of in the combat zone internally fixed fractures	47 patients with 50 fractures (14 hip, 14 forearm, and 14 ankle)	1 infection	MRSA
Burns et al.[279]	2003–2007	N/A	N/A	Does large zone of injury impact flap coverage	67 grade IIIB tibia fractures	None described	

N/A, not available.

TABLE 2. Gustilo Fracture Classification System and Associated Infection Rates

Gustilo Fracture Grade	Characteristics	Rates of Infection[32,169,191–193]
Grade I	Puncture wound <1 cm	0–2%
	Minimal contamination	
	Minimal soft tissue damage	
Grade II	Laceration >1 cm but <10 cm	2–5%
	Moderate soft tissue damage	
	Adequate bone coverage	
	Minimal comminution	
Grade IIIA	Laceration >10 cm	5–10%
	Extensive soft tissue damage	
	Adequate bone coverage, segmental/ severely comminuted fractures, or heavily contaminated wounds	
Grade IIIB	As a Gustilo grade IIIA injury, but with periosteal stripping and bone exposure	10–50%
Grade IIIC	Any open fracture with vascular injury requiring repair	25–50%

* Tibial fractures are associated with twice the infection rate of other bone.

ratory support; may be supplemented with surgical assets (Level IIb). Role 3—combat support hospital (CSH, US Army), Air Force theater hospital (AFTH, US Air Force), or casualty receiving ships (US Navy); full inpatient capacity with intensive care units and operating rooms. Role 4—regional hospital (Landstuhl Regional Medical Center, Germany) or USNS hospital ships (US Navy), typically outside of the combat zone; general and specialized inpatient medical and surgical care. Role 5—care facilities within United States, typically tertiary care medical centers.

METHODS

A MEDLINE search using PubMed from the US National Library of Medicine National Institutes of Health was performed using the key words "extremity," "orthopaedics," "military," "combat," "infection," "prevention," "osteomyelitis," "negative pressure wound therapy," "fixation," "irrigation," "debridement," "antimicrobial," "oxygen," "culture," "bacterial," "fungal," and "wound infection" with an emphasis on June 2007 through January 1, 2011. We also cross-referenced published bibliographies for additional manuscripts. In addition, we analyzed ongoing research projects with data published in abstract form or preliminary draft manuscripts for inclusion in the guidelines.

EPIDEMIOLOGY/MICROBIOLOGY OF WOUND COLONIZATION AND INFECTION

The primary complication associated with combat-related extremity injuries is infection. Approximately 15% of patients develop osteomyelitis, and ~17% of those infections relapse or recur.[10] Many of the traditional host factors associated with increased risk of extremity injury infections are not present in young, healthy military personnel.[18] Therefore, infections are likely to be related to the mechanism of injury, presence of orthopedic devices, fracture severity (grade), site of injury, antimicrobial agents received, infection prevention strategies employed, surgical care, environmental contamination, and infecting pathogens, especially those that are resistant to antimicrobials.[10,19–22] The bacteria infecting these wounds have included multidrug-resistant (MDR) bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, extended-spectrum β-lactamase-producing *Klebsiella* species and *Escherichia coli*, and methicillin-resistant *Staphylococcus aureus* (MRSA).[10,19,20] Although initial infections are often complicated by gram-negative pathogens, many of the late relapses are gram-positive bacteria, commonly methicillin-sensitive *S. aureus* and MRSA.[10,19,20]

POSTINJURY ANTIMICROBIALS

The nature of combat-related extremity injuries results in gross contamination of the wound along with anatomic and physiologic derangement of the local tissue. In addition, there are likely systemic immune alterations from the severe trauma complicating the patient's ability to control infection. Therefore, antimicrobial activity through systemic, and possibly local, delivery is required to prevent subsequent infections.

Timing of Antimicrobials

The current recommendation by the United States for tactical combat casualty care (TCCC) is rapid delivery of oral or intravenous antimicrobial therapy at the point-of-injury. This is primarily based upon expert opinion with limited supporting military data (Table 3).[23–27] Delivery of antimicrobials within a 3-hour window for limb soft tissue injuries was associated with fewer infections in comparison with those who received antimicrobials after 3 hours during the Falklands Campaign in 1982.[28] During the 1973 October War in Israel, the low rates of infections were attributed to casualties on the battlefield receiving antimicrobials within 30 minutes to 60 minutes of injury.[29] During the war in Afghanistan, the British military did not reveal that the timing of antimicrobials was related to infection prevention, but all antimicrobials were delivered soon after injury.[21] T3

Guidelines have recommended initiation of antimicrobials as soon as possible.[16,30] Retrospective civilian studies have not shown substantial differences in rates of infection based upon timing of the delivery of antimicrobial agents, but timing is typically defined by 3 hours and 6 hours, which might not correlate with the casualty taking the antimicrobial themselves or being provided by a medic near the time of injury.[31] One civilian study noted a higher infection rate (7.4%, 49 of 661 patients) if antimicrobials were given after 3 hours versus a lower infection rate (4.7%, 17 of 364) when antimicrobials were given within 3 hours.[32] However, this was not confirmed in other large studies, and care must be taken in general when comparing civilian and military trauma as the mechanism of injury can vary dramatically (i.e., motor vehicle crashes vs. blast injuries).[33–35] In addition, one of these studies was limited by lack of follow-up because many patients with grade IIIb and IIIc fractures being transferred to tertiary care hospitals for definitive management.[33]

TABLE 3. Relationship Between Timing of Postinjury Antimicrobial Delivery and Subsequent Infection Rate

Author	Year	Study Type	No. of Patients	Time to Antimicrobial Initiation	Infection Rates of Early vs. Late Antimicrobial Timing	Significant Difference
Patzakis an d Wilkins[32]	1989	Civilian, retrospective	1,104 (fractures)	≤3 h	4.7% (17 wounds of 364 open fractures) vs. 7.4% (49 wounds in 661 open fractures)	Yes
Al-Arabi et al.[33]	2007	Civilian, prospective	133	<2, <4, and <6 h	<2 h (9.2%) (6 of 65 patients) <4 h (2.2%) (1 of 45 patients) <6 h (0%) (0 of 14 patients) >12 h (100%) (2 of 2 patients)- surgery and antimicrobials delayed past 24 h	No, $P = 0.26$
Dellinger et al.[34]	1988	Civilian, prospective	240	≤3 h	16% (29 of 183 patients) vs. 17% (8 of 47 patients	No
Jackson[28]	1984	Military, retrospective soft tissue extremity injuries	49	≤3 h	0% (0 of 17 patients) vs. 28% (9 of 32 patients); 2 of 11 treated between 4 and 6 h became infected and 4 of 7 treated between 7 and 9 h became infected	None provided

Animal studies have shown that the earlier antimicrobials are provided, the more effective they are at preventing infections, especially in the first couple of hours after injury[36–41] (Joseph C. Wenke, personal communication).

Antimicrobial Agents of Choice

The choice of antimicrobials was selected based upon a review of prospective and retrospective clinical trials taking into consideration the bacteria likely associated with wound contamination in the combat zone (Table 4). In the previous guidelines, we recommended the use of the first-generation cephalosporin cefazolin because of its antibacterial coverage of likely infection pathogens and its use as the standard of care in the United States for extremity trauma.[16] This has remained the therapy of choice, without enhanced anaerobic or aerobic gram-negative bacterial coverage. In addition, dose modification and methods of delivery are outlined more specifically in this updated guideline.

Antimicrobials initially provided by the surgeon are selected to eradicate virulent bacteria likely inoculated into the wounds at the time of injury to prevent local and systemic infection. Yet, multiple studies reveal that the bacteria contaminating open fractures at the time of injury are not the same bacteria cultured from infected open fractures after debridement.[42–46] Instead, these infections are thought to be caused by hospital-acquired bacteria.[22,42,47] Nosocomial infections with late onset wound infections were well described during World War II.[48–50] In the Korean War, pathogens infecting wounds within 8 hours of injury included *Clostridium* species along with gram-positive and gram-negative pathogens.[51] In addition, wounds appeared to have varying types of bacteria isolated from them over the course of a serviceman's hospitalization, but infections only occurred when wounds had necrotic tissue remaining.[52] During the Vietnam War, there was a transition over 5 days from an even mix of gram-negative and gram-positive bacteria within wounds at the time of injury to primarily gram-negative pathogens, notably *P. aeruginosa*, despite (or because of) broad spectrum antimicrobial therapy (typically penicillin and streptomycin) active against the bacteria initially found in the wound.[53] Notably, wound cultures did not always correlate with matching blood cultures, and infections primarily occurred in wounds with necrotic tissue remaining. Bacteria recovered in Japan, ~7 days after injury, had a predominance of *P. aeruginosa* and *S. aureus* followed by *Enterobacter* spp. In addition, when comparing the susceptibility patters of these organisms over time, it appeared that antimicrobial resistance increased over the course of their hospitalization.[54] The presence of these pathogens remained in wounds upon arrival in the United States.[55] During the wars in Iraq and Afghanistan, one study found that cultures from wounds at the time of injury reveal a predominance of gram-positive bacteria without MDR gram-negative rods.[56] Overall, numerous wounds appear to be colonized and possibly infected upon reaching care within the United States or England with the burden of MDR pathogens increasing over time, as appears to have occurred in previous wars.[10,19–21,57,58]

The International Committee of the Red Cross (ICRC) recommends intravenous penicillin for compound fractures, amputations, and major soft tissue wounds.[59] The British military has traditionally provided penicillin-based regimens at the initial time of surgery, including intravenous amoxicillin/clavulanate for abdominal injuries; however, there is debate as to the ideal agent.[21,60] The Israelis' management of injuries (predominantly from blasts) has included a combination of cephalexin and metronidazole intravenously followed by ortal therapy.[61]

Among civilian trauma care, a Cochrane review indicated that antimicrobials were protective against early infection compared with no antimicrobials (relative risk 0.41, 95% confidence interval 0.27–0.63; absolute risk reduction of 0.08, 95% confidence interval 0.04–0.12; and number needed to treat of 13).[62] This effect was attributed to the activity of β-lactams antimicrobials against streptococci and staphylococci. The Eastern Association for the Surgery of Trauma guideline committee concluded that antimicrobials were useful, but further work was needed, especially regard-

TABLE 4. Randomized or Prospective Studies of Antimicrobial Prophylaxis in Open Fracture

Reference	Year	No. of Patients	Fracture Type	Grade	Wound Management	Antimicrobial Strategy	Duration of Antimicrobial	Outcomes	Comments
Patzakis et al.[31]	1974	310	Long bones	No	Primary suture	Cefazolin vs. penicillin plus aminoglycoside vs. placebo	10–14 d	Cefazolin (2%) < penicillin plus aminoglycoside (10%) < placebo (14%)	
Bergman[280]	1982	180	Mostly long bones	Yes	Primary suture	Dicloxacillin vs. penicillin vs. placebo	2 d	2 deep infections in placebo group only	12 grade II and III treated with dicloxacillin, 10 with benzyl penicillin, and 13 with saline
Benson et al.[259]	1983	82	Long bones	No	Variable	Clindamycin vs. cefazolin	5 d	Clindamycin = cefazolin (~3%)	
Sloan et al.[91]	1987	85	Distal phalanx	No	N/A	Cephradine	1 d vs. 5 d	5 d = 1 d	
Braun et al.[281]	1987	100	Long bones	No	Not reported	Dicloxacillin vs. placebo	10 d	Dicloxacillin (2%) < placebo (12%)	
Johnson et al.[282]	1988	86	Tibia	Yes	Delayed closure	Cefazolin vs. cefotaxime	Variable	Cefazolin (24%) = cefotaxime (19%)	Some more Gram negative failures in cefazolin and Gram positive in cefotaxime
Dellinger et al.[34]	1988	248	Long bones	Yes	Delayed closure	Cefonicid 1 d vs. 5d vs. cefamandole 5d	1 d vs. 5 d	Cefonicid 1 d = 5 d—cefamandole 5 d (12–13%)	
Peacock et al.[283]	1988	87	Hand	No	N/A	Cefamandole intravenous then oral cephalexin vs. placebo	4 d	Antimicrobials (0%) = placebo (2.1%)	
Dickey et al.[284]	1989	67	All injuries	No	Closed	24-h cefazolin vs. no therapy	1 d	1 infection each arm	Low-velocity gun shots, 29 lost to follow-up
Swiontkowski[285]	1989	60	Lower extremity	Yes	Prospectively varied	All received cephalosporin plus aminoglycoside	Variable	None	
Robinson et al.[164]	1989	89	Lower extremity	Yes	Delayed closure	All received cefoxitin plus aminoglycoside for grade III	Variable	None	
Suprock et al.[286]	1990	91	Finger	No	N/A	3 d Gram positive coverage vs. placebo	3 d	Antimicrobials (9%) = placebo (9%)	
Hansraj et al.[287]	1995	100	All injuries (gunshot wounds)	No	N/A	Ceftriaxone vs. cefazolin	2 d	Ceftriaxone = cefazolin (0%)	30% lost to follow-up
Knapp et al.[288]	1996	186	Mostly long bones	No	Delayed closure	Intravenous cephapirin plus gentamicin vs. ciprofloxacin	3 d	2 infections in each arm	Low-velocity gunshot wound
Vasenius et al.[70]	1998	227	Mostly long bones	Yes	Delayed closure	Clindamycin vs. cloxacillin	3 d or until closed	Clindamycin (9%) < cloxacillin (20%)	Most infections were in grade I fractures

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TABLE 4. Randomized or Prospective Studies of Antimicrobial Prophylaxis in Open Fracture (continued)

Reference	Year	No. of Patients	Fracture Type	Grade	Wound Management	Antimicrobial Strategy	Duration of Antimicrobial	Outcomes	Comments
Carsenti-Etesse et al.[42]	1999	616	Leg grade I-II	Yes	Delayed closure	Pefloxacin vs. cefazolin plus oxacillin	1 d vs. 5 d	Pefloxacin (7%) = cefazolin plus oxacillin (8%)	More Gram negative infections in cefazolin group but more *S. aureus* in pefloxacin group
Sorger et al.[76]	1999	71	Mostly long bone	Yes	Delayed closure	Cefazolin plus gent 5 mg/kg q 12 h vs. 6 mg/kg q24 h	48 h after each operation until wound closed	Q 12 h gent (13.6%) = q 24 h (5.4%)	
Moehring et al.[130]	2000	67	Leg grade II-IIIb	Yes	Delayed closure	"Conventional antimicrobials" vs. same plus tobramycin beads	Until closed	"Conventional antimicrobials" (5%) = same plus tobramycin beads (8%)	Conventional antimicrobials not controlled, 20% randomization error
Patzakis et al.[65]	2000	171	Mostly long bones	Yes	Delayed closure	Ciprofloxacin (intravenous or oral) vs. cefamandole plus gentamicin	3–8 d	Ciprofloxacin- cefamandole plus gentamicin	>12 h to antimicrobials, >24 h to OR. High infection rates in grade III fractures
Stevenson et al.[289]	2003	193	Phalynx	N/A	N/A	Flucloxacillin vs. placebo		3% vs. 4% infection	

N/A, not available.

ing grade IIIb fractures. They recommend systemic antimicrobials directed at gram-positive organisms with additional gram-negative coverage for grade III fractures. They indicated that fluoroquinolones offer no advantage over cephalosporin plus aminoglycosides with the possibly association of excess harm.[30,63] The Surgical Infection Society concluded that current studies for determining antimicrobial recommendation suffer from methodological and statistical flaws, older publications and studies not adequately reflecting the bacterial resistance, or the available antimicrobial agents used today.[64] These guidelines do not support the addition of enhanced gram-negative coverage with an aminoglycosides for grade III fractures.

Enhanced Gram-Negative Coverage

A major area of controversy in the selection of postinjury antimicrobials involves the role of additional gram-negative coverage for grade III fractures. Prospective studies with ciprofloxacin have shown no improved outcomes and actually worse outcomes for grade III fractures in comparison with cefamandole and gentamicin.[65] There is also in vitro and animal data which has associated fluoroquinolone use with impaired fracture healing.[66,67] The role of additional aminoglycoside coverage was only assessed prospectively in one study evaluating no antimicrobials, penicillin plus streptomycin, and cephalothin for 10 days.[31] That study did not describe the grades of fractures. There was a 13.9% infection rate in the placebo group (11 of 79 wounds), 9.7% in the penicillin plus streptomycin group (9 of 92 wounds), and 2.3% in the cephalothin group (2 of 84 wounds) (no significant difference between placebo or penicillin plus streptomycin group [$p = 0.45$] but significant difference between cephalothin and placebo and penicillin plus streptomycin groups [$p < 0.05$]). Cultures were wound swabs but were not obtained for clinical evidence of infection. The bacteria in the penicillin plus streptomycin had the highest rate of recovered resistant pathogens after therapy. Interestingly, the cultures from the placebo group for pathogenic bacteria remained stable around 40% before antimicrobials until wound closure, whereas for the penicillin plus streptomycin group, pathogenic bacteria increased from 32% before surgery to 83% at wound closure. For the cephalothin group, bacteria recovery was 33% before antimicrobials and negative at the final wound closure culture. Although this study by Patzakis et al. has been referred to as prospective research supporting the use of enhanced gram-negative coverage, the reported results do not appear to support this recommendation. The data supporting the recommendations for aminoglycosides are typically cumulative studies that includes this prospective study just mentioned in combination with a retrospective study in which a combination of cefamandole plus tobramycin in 109 wounds had five infections (4.5%).[32,68,69] An additional argument that has been made is based upon a prospective study comparing clindamycin versus dicloxacillin with high rates of failure with grade III fractures.[70] The authors propose that success rates could be improved by the addition of gram-negative coverage. Notably, an evaluation of possible infecting pathogens (removing likely skin patho-

gens such as diphteroid, micrococcus, *Bacillus* species, and *Streptococcus viridians*) recovered from initial wound swabs were 35% gram-negative and 65% gram-positive. Clinical failure included pathogens that should have been adequately covered with initial regimens. Overall, these studies support adequate irrigation and debridement as primary therapy, with antimicrobials as adjuvants relegating later infections to primarily nosocomial transmission with antimicrobial potentially selecting pathogens.

Given the MDR nature of the gram-negative bacteria found to be subsequently infecting combat casualties' injuries after the current antimicrobial regimens were used in the combat zone (e.g., cefazolin and levofloxacin or aminoglycoside), it is currently not clear whether the use of fluoroquinolones with enhanced gram-negative activity or aminoglycosides is resulting in the selection of these resistant pathogens, as shown in the civilian literature. Although not rigorously evaluated, data derived from the Yom Kippur War indicated that overly broad spectrum antimicrobial agents led to the development of infections with resistant bacteria.[71] Those authors proposed that the severity of combat trauma wounds and contamination "leads toward the temptations to 'sterilize' the wound with massive doses of antimicrobials and favors a false security with less reliance on good surgical technique."

If an aminoglycoside is to be used to "enhance gram-negative coverage," it will be a challenge to determine which agent to use based upon the varying resistance profiles of the gram-negative rods being recovered from combat-related extremity injury infections.[72–75] For *P. aeruginosa* and *E. coli* isolates from patients managed on the US Comfort, 94% were amikacin susceptible, and only 40% were susceptible to gentamicin or tobramycin.[74] For *Enterobacter* species, 78% were gentamicin susceptible, whereas 40% were amikacin or tobramycin susceptible. If aminoglycosides use is implemented, daily dosing appears adequate, at least for gentamicin; however, higher doses might be required in a severe trauma patient, especially with these MDR bacteria.[76]

Addition of Penicillin for Dirty Wounds

Justification for penicillin therapy has traditionally focused on gas gangrene infections or *Streptococcus pyogenes* (Group A streptococci). During World War I, there was a 5% incidence of gas gangrene, with 28% mortality; during World War II, the incidence ranged between 0.3% and 1.5%, depending upon the combat zone, with 15% mortality.[77–80] During the Korean War, there were no reported cases of mortality as a result of this complication.[81] This decrease was largely attributable to decreasing the time from injury to definitive care and adequate surgical debridement, not specific antimicrobial therapy. In the current era, there is some controversy about the use of penicillin after trauma. The Eastern Association for the Surgery of Trauma practice management guidelines for civilian injuries recommends high-dose penicillin when there is concern for fecal/clostridial contamination such as in farm-related injuries.[30,63] However, penicillin therapy is discouraged by the Surgical Infection Society regardless of the mechanism of injury.[64] The primary reason for not providing penicillin is the rarity of gas gangrene seen among wounds, especially combat-related extremity injuries in wartime as a result of aggressive surgical management and delayed primary closure. To date, no cases have been described in Iraq or Afghanistan. Of increasing concern is the increase in in vitro resistance to penicillin in *Clostridium* species and limited animal data that reveal no improved outcomes with antimicrobial therapy for gas gangrene in comparison with untreated controls.[21,56,77,82,83] Finally, other antimicrobials, typically provided during extremity wound care by physicians, have adequate Group A streptococci coverage limiting the utility of additional penicillin coverage.

Point-of-Injury Tactical Combat Casualty

A panel of military trauma experts published a list of antimicrobials that were recommended as part of TCCC or care provided at the point-of-injury. These include oral moxifloxacin and intravenous/intramuscular ertepenem or cefoxitin.[24,25,84] Although the ICRC, the British military, and the Israeli military recommend various antimicrobials for combat-related injuries, these are not designed to be given at point-of-injury by the patient or the medic but upon evaluation by definitive medical personnel often times during the medical evacuation flight.[21,59–61]

The core issues surrounding the determination of the ideal point-of-injury field antimicrobial is multifactorial. As addressed in articles assessing point-of-injury antimicrobial agent, the goal is to include agents that are active against the likely infecting pathogen for the body part injured along with agents that are stable and able to be delivered in a reasonable manner on the battlefield without possible adverse events to the patient.[24,25,60,84] A recent study evaluating point-of-injury antimicrobials by Army Rangers did not appear to show a clear infection prevention benefit, although the numbers were small. Of note, no increase in colonization or infection with MDR bacteria was noted nor were there medication toxicities reported.[27] There are clear arguments for choosing an agent with focused antibacterial spectrum of activity; however, it appears the antimicrobials recommended by the TCCC Committee are not causing harm and might be beneficial.

Dosing of Antimicrobials

To better optimize antimicrobial pharmacodynamics and pharmacokinetics, higher doses of antimicrobials are being recommended for perioperative antimicrobials to prevent surgical site infection.[85,86] Most recommendations for perioperative antimicrobials are for a cefazolin dose of 2 g with some recommendations being weight-based: 1 g for those ≤80 kg (176 lbs), 2 g for those 81–160 kg (177–352 lbs), and 3 g for those >160 kg (>352 lbs).[87,88] The package insert for cefazolin does recommend higher dosing (1–1.5 g every 6 hours) for severe, life-threatening infections with dosing up to 12 g per day being given.[89] Given the volume of distribution in a trauma patients and the size of the typical US servicemember, a 2 g dose of cefazolin is recommended and appears to be safe.

Duration of Antimicrobials

The ICRC recommends a total of 5 days of antimicrobial therapy after injury, which is similar to the Israeli recommendation of 5 days after missile injury.[59,61] Per the ICRC, if redebridement is performed instead of delayed primary closure, antimicrobials should be stopped if there are no signs of infection or local inflammation. If patients present after 72 hours or are injured as a result of antipersonnel landmines, then the addition of metronidazole in an intravenous form for 48 hours followed by oral therapy until delayed primary closure is suggested. Most authors and guidelines focusing on civilian injuries recommend 24 hours to 72 hours of postinjury antimicrobial therapy depending upon severity of injury, with shorter durations for grade I fractures and longer durations for grade III fractures.[30,63,64,90] Prospective and some retrospective studies have revealed therapy as short as 1 day may be as effective as the traditionally recommended 3 days to 5 days of therapy and 3 days better than 5 or more days.[32,34,43,91] There also are data suggesting that prolonged courses of antimicrobials are associated with systemic infections with MDR bacteria.[92,93] In addition, 72 hours is typically the time in which wounds are surgically reevaluated in a combat setting, allowing antimicrobials to be discontinued if there is no evidence of ongoing infection.

Redosing of Antimicrobials

In addition to higher dosing, repeat dosing before the 2-hour to 4-hour interval, typically recommended for surgical site infection prevention, should occur if blood loss exceeds 1,500 mL to 2,000 mL.[86,94–96] Although the literature does not necessarily apply to the very large volumes of blood loss and potential whole blood requirements among casualties of war, it is reasonable to redose cefazolin when there is large volume blood loss and possible large volume fluid resuscitation.[97–101]

Alternate Routes of Systemic Antimicrobial Delivery

Methods to deliver antimicrobials is challenging during combat operations due to logistical constraints associated with supplying medications, storage of medications, and obtaining and maintaining adequate venous access. The use of intraosseous (IO) delivery of fluids or analgesia has been recommended as part of TCCC; however, in the TCCC guidelines for point-of-injury antimicrobials for those unable to take oral agents (shock, unconscious, or penetrating torso injures), the recommendation is for delivery by intravenous (IV) or intramuscular (IM) route.[24,25,102] IO antimicrobial delivery has not been systematically studied in military populations or trauma patients.[103,104] In animal studies, those antimicrobials that are highly protein bound have been associated with lower serum concentrations with IO delivery than IV delivery.[105] Both cefazolin and ertapenem are highly protein bound antimicrobials.[106,107] IM delivery has also not been studied in military or trauma patient populations but has been used and FDA approved for cefazolin and ertapenem.[107–109] An animal study assessing a first-generation cephalosporin revealed that highest peak levels were achieved by IV push; however, IM route was associated with sustained serum levels of drug.[110] As bone to serum penetration ratio for ertapenem is ~0.15 and for cefazolin it is 0.25, this low concentration of antimiocrobial would limit IO or IM delivery, especially with severely ill patients and resistant bacteria.[111–114]

Local Antimicrobial Delivery

Local delivery of antimicrobials as a powder or solution was discouraged by Alexander Fleming in 1919 while he served in the British Army.[115] During World War II, antimicrobials were shown to be more effective if given systemically than through local delivery.[116–118] During the Vietnam War, topical therapy was not broadly implemented, although it appeared to be associated with lower rates of bacteria in wounds in some animal studies.[119–124]

During the current wars in Iraq and Afghanistan, there have been a number of reports of the utilization of local delivery of antimicrobials through beads or bead pouches, but limited comparative trials and no prospective trials are available to support this use.[125] One retrospective study comparing antimicrobial bead pouch to negative pressure wound therapy (NPWT) revealed fewer infections with the antimicrobial bead pouch.[126]

There is extensive use of local delivery of antimicrobials through beads, which might benefit in clearing infections due to high local drug concentrations, especially those associated with biofilms or bacteria resistant to standard levels of antimicrobials.[127,128] Traditionally, vancomycin and tobramycin have been the agents of choice, but other agents such as colistin have been pursued because of the MDR nature of infecting pathogens.[129] A randomized, prospective study of civilian open fractures with 67 patients and 75 open fractures revealed 2 of 24 (8.3%) with antimicrobial beads alone developed an infection in contrast to 2 of 38 (5.3%) of those treated with conventional systemic antimicrobial therapy.[130] A large retrospective study of open extremity fractures revealed statistically significant reduction in infections in those patients receiving local delivery of antimicrobials (tobramycin) versus those receiving only systemic therapy (12% vs. 3.7% [$p < 0.01$]).[131] In that study, the patients with impregnated beads had their wounds closed earlier, introducing a potential bias into the study conclusions. The use of antimicrobial bead pouches has also been retrospectively assessed in combination with intramedullary nails for grade II, IIIa, and IIIb tibia fractures.[132] Of 50 patients who received the antimicrobial bead pouches, only 2 developed an infection, in contrast to four infections in the 25 patients who did not receive the pouches.

In animal models, local delivery of antimicrobials appears promising.[133] However, there is possible toxicity to osteoblasts associated with local delivery of some antimicrobial agents.[67] Of note, antimicrobials used in combination with NPWT appears effective; however, NPWT also pulls antimicrobials out of the wound and reduces their effectiveness when compared with standard bead pouch (see NPWT section below).[134] The practical use of bead pouches during aeromedical transport and frequent serial debridements remains a difficult technical challenge. It also appears that earlier delivery of local antimicrobials with earlier surgery

might improve outcomes.[135] Moreover, the appearance of wounds might differ with local delivery of agents, especially silver, potentially impeding clinical diagnosis of wound infection.

DEBRIDEMENT AND IRRIGATION

The gross contamination of wounds at the time of injury necessitates adequate irrigation and debridement to prevent ongoing bacterial replication. In addition, the presence of devitalized tissue is an ideal culture media, which must be adequately controlled to prevent subsequent infections.

Irrigation Fluid Additives

No combat-related extremity injury studies have evaluated the role of additives to irrigation fluid for wound management, although one study assessing NPWT in a combat support hospital did describe the role of irrigation in wound management.[136] In addition, another study assessing minor wounds that did not require evacuation for surgical care revealed the primary importance of wound irrigation over antimicrobials.[137]

A large, multicenter collaborative project assessing various irrigation pressures and irrigation solutions of open fractures is under way and should provide insight into the ideal irrigation strategies.[138] This group performed a thorough review of the current irrigation literature. Preliminary data have revealed that low volume with castile soap might be beneficial.[139] The only prospective, randomized clinical trial was limited to a single institution with relatively small numbers of enrolled participants.[140] Patients were randomized to irrigation with a bacitracin solution or a nonsterile castile soap solution with overall findings indicating that bacitracin was no better than nonsterile castile soap but was associated with a possible increased risk of wound-healing problems. Another study limited to lacerations revealed no difference between normal saline and sterile water.[141] Reviews and surveys of provider practice patterns indicate no clear support for additives into irrigation fluids, supporting the role of normal saline or sterile water and even potable water if the other fluids are not available.[90,142–144] Animal studies have also supported this conclusion.[145,146]

Volume of Irrigation Fluid

Although not the primary focus of a study evaluating the use of NPWT performed on casualties in Iraq, the use of pulsatile jet irrigation with at least 3 L of saline was shown to be very successful overall management strategy that decreased combat-related injury infection rates.[136] No clear studies have proven the efficacy of the commonly used volumes for various grades of fractures (3 L for grade I fractures, 6 L for grade II fractures, and 9 L or more for grade III fractures), but this appears to be standard of care throughout the world.[144] In animal models, it has been shown that greater volumes removes more bacteria and that greater volumes are likely needed in removing debris when low-pressure irrigation is used.[147]

Pressure Employed to Deliver Irrigation Fluids

Irrigation fluid pressure includes gravity flow (1–2 psi [pounds per square inch] typically obtained by hanging the irrigation fluid bag 6 feet to 8 feet above the ground), low-pressure irrigation (5–10 psi), or high-pressure irrigation (>20 psi), although these definitions are not standardized. Overall, the reasoning behind which pressure provides the best patient outcomes was well outlined in a recent review of the subject. It showed that higher pressure initially cleans the wounds very well, but tissue and bone damage along with rebound bacterial colonization noted 24 hours after initial irrigation limits its overall positive impact.[90,138] It is anticipated that the Fluid Lavage and Open Fracture Wounds multicenter, randomized trial will provide adequate data to answer this clinical delima.[138] Pilot data from the Fluid Lavage and Open Fracture Wounds study does demonstrate a trend toward more wound complications with high-pressure devices.[139]

Timing of Irrigation

Currently, there are limited data available regarding the timing of irrigation fluid delivery, as it is often lumped with routine surgical care. It is vital to have this information to show whether delivery of earlier field irrigation with point-of-injury field antimicrobials might improve infectious complications in a combat setting when evacuation is not possible.[137] Animal studies have shown that earlier irrigation improves bacterial clearance as irrigation within 3 hours decreased bacteria counts by 70% in contrast to 52% if irrigation was delayed to 6 hours or 37% if delayed to 12 hours.[148]

Pre- and/or Postdebridement Bacterial Wound Cultures

A study in Vietnam pertaining to cultures of wounds collected ~7 days from injury revealed that an initial negative culture was associated with 32% of patients developing a subsequent infection and a positive culture was associated with 50% of patients becoming infected.[149] Other military studies from the Korean War, the Vietnam War, and other conflicts have described similar bacterial patterns recovered from wounds and nosocomial infections.[51,52,54,55,71,150,151]

There are limited reports of wound cultures from casualties at the time of injury in Iraq or Afghanistan. A single study in Iraq describing 15 of 24 extremity injuries in US Servicemen found a predominance of gram-positive bacteria including occasional MRSA but recovered no MDR gram-negative bacteria at the time of injury.[56] A limitation of this study is that patients were not followed for subsequent infections. A number of studies have assessed the role of MRSA soft tissue infections in the combat zone, including extremity injuries, but the wounding pattern and long-term complications have not been characterized.[152–155] After leaving the combat zone, patients are presenting to US military hospitals with a much higher rate of MDR gram-negative bacteria colonizing and infecting wounds.[10,19–21] It is remarkable that gram-positive pathogens are often found later in a patient's hospital course and typically after eradicating patient's initial colonization or infection with gram-negative

pathogens. It is not clear whether these gram-positive bacteria were the same pathogens initially seen at the time of injury or reflective of nosocomial transmission.[10] A recent study using tissue biopsy culture characterization of wounds reports that 69% of 242 wound biopsies from 34 patients had no growth at the time of presentation to a US military treatment facility.[58] The most commonly recovered pathogen in this study was *A. baumannii*, and of note, the incident colonization of wounds increased when examined serially over the course of 3 weeks. This study did not provide details concerning quantity of bacteria in 1 cm^3 of tissue biopsied or infections associated with the wounds that underwent biopsy. Another study of British soldiers with mangled extremities revealed that bacteria initially recovered from injuries that were not the same as those later infecting wounds, but the presence of bacteria in general was possibly predictive of future infections.[21] In addition, ~25% of war wounded patients admitted to Walter Reed Army Medical Center developed new colonization with MDR gram-negative bacteria during their hospitalization, although this study did not evaluate the infection rates.[75]

The Surgical Infection Society guideline for prophylactic antimicrobial use provides a summary of the limited role of cultures associated with open fractures.[64] Available civilian data support similar findings to the military, with gram-positive bacteria predominating at the time of injury and a transition to gram-negative bacteria causing ultimate infection. In addition, empiric therapy can modify the bacteria recovered.[32,42,44] Pre- or postdebridement cultures do not appear to be consistently predictive of infection or infecting bacteria, and initial choice of early antimicrobial agents can result in bacteria that escape the initial spectrum of activity.[32,42,44–46,156–163] Although postdebridement cultures have been reported to be more predictive of infection in some studies, they are not always reflective of the infecting bacteria.[43,44] Some studies have supported cultures obtained 1 day after debridement that reveal the same pathogen as previously recovered are reflective of failure of debridement and subsequently high risk of infection.[164] Additional studies have also looked at correlation with bacterial counts, notably $>10^5$ bacteria per gram of tissue, but these studies did not appear to substantially correlate with infections.[43,165–168] In addition, some studies have supported a standardized approach to culture to predict closure success.[169] At this time, we are unable to predict which patients will go on to develop infection based upon wound cultures alone. Therefore, novel diagnostic platforms are required to describe the bioburden in the wound.[170]

Removal of Retained Metal Fragments

Many of the weaponry systems used in combat can result in numerous fragments lodged into the body with associated tissue damage.[171–176] The fragments can be associated with the deposition material that impact infectious complications.[177–179] Two strategies, one in Gaza City and the other along the Afghan border, have been employed for nonoperative management of retained metal fragments in the following list of injuries: soft tissue injuries (no fractures, no major vascular involvement, and no break of pleura or peritoneum), small wound entry or exit maximum dimensions, wounds not frankly infected, and wounds not caused by mines.[61,171] Management in both cases included cleaning and dressing the wounds and administration of antitetanus immunoglobulin and toxoid, penicillin IM/IV for 1 day and then orally for the next 4 days, or cephalexin and metronidazole for 2 days intravenously and then orally for 3 days. Minimal complications occurred with these management strategies.

Management of victims of suicide bombers has included small fragments remaining in patients, but no clear management strategy for fragment removal or management strategies to prevent infections has been described. Hepatitis B virus prophylaxis, due to reports in Israel of hepatitis B virus recovered from bomber's bone fragment, has been recommended for those not previously vaccinated, due to theoretical risk of transmission.[180–182] The decision not to remove small fragments has been questioned based upon a pediatric study associated with a suicide bomber in which the retained fragments became symptomatic.[183] However, the application of this patient population and injury pattern might not equate to the military. Studies of minor gunshot wounds with fragments remaining have also shown small infection rates when managed using similar criteria to the above.[184]

SURGICAL WOUND MANAGEMENT

Timing of Surgical Management

Historically, evacuation times have continued to decrease from 11 hours during World War II to 4 hours during the Korean War, to 3 hours during the Vietnam War, and to 1 hour to 3 hours in Iraq and Afghanistan.[81,185,186] Traditionally, it has been recommended that open fractures undergo operative procedures within 6 hours of injury to mitigate infectious complications. Data assessing outcomes based on time to procedures are limited for combat casualties (Table 5). Among those with extremity soft tissue injuries during the Falkland Campaign, there were 2 septic patients among 20 who underwent surgery within 6 hours in contrast to 7 of the 29 patients treated after 6 hours. Nine of those 29 went to surgery after 15 hours, 3 of whom became septic.[28] The US military experience in Somalia revealed 14 of the 16 casualties that developed infection were treated either outside of Somalia and/or after 6 hours, but long-term infectious outcomes were not described.[187] During the war in Afghanistan, an evaluation of British military personnel with mangled extremities revealed that time to surgery had no impact on infectious complications, but this group of wounded were all evacuated rapidly to surgical care.[21] There is military experience with delayed surgical interventions during humanitarian missions, with good outcomes being reported in host nation patients.[188]

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Wound debridement and irrigation removes foreign material, blood clots, bone fragments, and marginally vascularized tissues, which are penetrated poorly by antimicrobials and provide a good medium for bacterial proliferation.[64] Civilian guidelines recommend that rapid surgical debridement is the primary treatment, and antimicrobials only adjuvant therapy, in the prevention of infection in open fracture management.[30,90,189] A study by Friedrich[190] has historically

TABLE 5. Relationship Between the Time to Debridement and Subsequent Infection Rates

Author	Year	Study Type	Fractures (n)	Time to Debridement	Infection Rates of Early vs. Late Debridement	Significant Difference
Jackson[28]	1984	Military, retrospective, soft tissue extremity injuries	49	0–3, 4–6, 7–9, 10–11, and >13	9% (1 of 11) vs. 11%(1 of 9) vs. 25% (2 of 8) vs. 10% (1 of 10) vs. 31% (4 of 1)	No <3 h or <6 h total data
Patzakis and Wilkins[32]	1989	Civilian, retrospective	1,104	12 h	6.8% vs. 7.1%	No?
Bednar and Parikh[290]	1993	Civilian, retrospective	82	6 h	9% vs. 3.4%	No
Kreder and Armstrong[194]	1995	Civilian, retrospective	56	6 h	12% vs. 25%	Yes
Kindsfater and Jonassen[163]	1995	Civilian, retrospective	47	5 h	7% vs. 38%	Yes
Skaggs et al.[291]	2000	Civilian, retrospective	118	6 h	2.5% vs. 6%	No
Harley et al.[195]	2002	Civilian, retrospective	215	13 h	8% vs. 7%	No
Rohmiller et al.[292]**	2002	Civilian, retrospective	390	8 h (average)	N/A	No
Taitsman et al.[293]**	2002	Civilian, retrospective	334	<8, 8–18, >8 h	N/A	No
Khatod et al.[294]	2003	Civilian, retrospective	106	6 h	16% vs. 20%	No
Ashford et al.[295]	2004	Civilian, retrospective	48	6 h	17% vs. 11%	No
Spencer et al.[296]	2004	Civilian, retrospective	115	6 h	10.1% vs. 10.8%	No
Noumi et al.[220]	2005	Civilian, retrospective	89	6 h	5.3% vs. 2.9%	No
Skaggs et al.[297]	2005	Civilian, retrospective	554	6 h	3% vs. 2%	No
Charalambous et al.[298]	2005	Civilian, retrospective	383	6 h	53% vs. 51% (overall infection) 8% vs. 8% osteomyelitis	No
Mathes and Brasher[299]	2006	Civilian, retrospective	891	6, 8, 12, 16, and 24 h	N/A	No
Naique et al.[300]	2006	Civilian, retrospective	73	6 h	7.1% vs. 16%	No
Al-Arabi et al.[33]	2007	Civilian, prospective	248	6 h	7.8% vs. 9.6%	No
Tripuraneni et al.[301]	2008	Civilian, retrospective	215	6, 6–12, 12–24, >24	10.8% vs. 9.5% vs. 5.6% vs. 0%	No
Pollak et al.[35]	2010	Civilian, retrospective	215	<5, 5–10, >10 h	28.0% vs. 29.1% vs. 25.8%	No
Brown et al.[21]	2010	Military, retrospective	74	<3, <6, <12 h	50% vs. 75% vs. 94%	Yes

N/A, not available.

been cited as the source for the "6-hour rule" for time to debridement of open fractures. An additional study evaluated 46 patients with grade II and III open fractures and found that 1 of 15 (7%) who underwent debridement in <5 hours from the injury became infected, whereas 12 in 32 (38%) became infected when debridement occur >5 hours after the initial injury.[163] Notably, grade III fractures comprised 33% of the <5-hour group and 53% of the >5-hour group. Multiple studies by Gustilo et al., as well as Patzakis et al., have shown that there is an increased risk of infection associated with an more severe Gustilo grade of open fractures and with delayed surgery.[32,191–193] Thus, the disproportionate number of severe fractures in the delayed debridement group could have skewed their results to favor early debridement. In addition, a small study of 56 open fractures in children showed increased infections when debridement was delayed more than 6 hours.[194] A more recent study looked at 248 open fractures and found infection rates of 7.8% and 9.6% when debridement occurred within 6 hours or >6 hours after injury, respectively ($p = 0.6$).[33] These data are in accordance with a larger study which showed that whether debridement occurred <12 hours or >12 hours after injury, the infection rate was not significantly different; 7.1% versus 6.8%.[32] Another study showed that the risk of an adverse outcome, deep infection or nonunion, was not increased by debridement or definitive treatment >13 hours from the time of injury.[195] A recent study of 315 severe high-energy extremity injuries revealed that time to debridement was not associated with infection rate (<5 hours, 28% infection rate [93 patients]; 5–10 hours, 29.1% infection rate [86 patients]; and >10 hours, 25.8% infection rate [128 patients]).[35] Interestingly, this study indicated that time to arrival at a definitive care trauma center was the most important factor associated with decreased infection rate.

Timing of Wound Closure

It is currently recommended that closure of wounds in combat environments be delayed, based upon lessons learned during prior wars and supported by recent conflicts and civilian literature.[59,136,196–198] However, wounds are still recommended to be closed at ~5 days if there is no evidence of infection, if it is technically possible. For vascular injuries, covering the artery with healthy tissue, to include flaps, is recommended.[12,13,199] If there is a need to reconstruct an artery within a large zone of injury, tunneling the bypass or repair through clean tissue planes has been recommended. The use of autogenous tissue is also better than prosthetic, but prosthetic may need to be used in patients who do not have appropriate veins to harvest.

There have been an increased number of civilian trauma centers evaluating early closure of wounds due to the findings that nosocomial bacteria are typically causing infec-

tious complications.[90,200–204] Two retrospective studies have reported immediate wound debridement and closure in open fractures.[200,201] A retrospective comparative review of early versus delayed closure in open fractures showed no difference in infectious complications with primary (2%) versus delayed (4%) closure.[202] A review in 2007 recommended primary closure if certain criteria are met: (a) debridement performed within 12 hours, (b) no skin loss primarily or secondarily during debridement, (c) skin approximation possible without tension, (d) no farmyard or gutter contamination, (e) debridement performed to the satisfaction of the surgeon, and (f) no vascular insufficiency.[205] Unfortunately, most military injuries are not compatible with this injury pattern and criteria.

Fracture Fixation Strategies

Staged fixation in combat injuries has emerged as the strategy of choice in the current conflicts.[16] Temporary external fixation has been commonly used as a bridge to definitive fixation with few significant complications.[206] Although a few selected cases of low-energy injuries have been safely internally fixed in the combat zone, it is still considered "ill-advised" in combat-related injuries.[207,208] The use of plaster and earlier internal fixation might be possible as evident by the British military experience.[21,188,209] In addition, there can be delays associated with femoral neck fractures >48 hours and talar neck fractures, which are consistent with civilian data.[210–214]

Because little data on combat-related femur fractures have been published in the past 4 years, the recommendation for intramedullary nailing is supported by civilian data.[215,216] Reamed intramedullary nailing of open femur fractures has been associated with infection rates of 1.8% to 5%.[217–219] Most infections in open femur fractures occur in grade III open injuries.[217,220] Based upon available literature on femur fractures, temporary spanning external fixation could be placed at Role 2b-3 with skeletal traction and Thomas's splint as alternatives. Conversion to definitive fixation at Role 4 remains controversial. Delayed conversion of external fixation to a reamed, locked intramedullary nail can be performed at Role 5 facilities after appropriate wound management.

Open tibia fractures typically have higher infection rates than open femur fractures when converted to internal fixation.[221,222] Despite these moderate infection rates, the intramedullary nailing of open tibia fractures after external fixation demonstrated significantly faster union and greater range of motion with less malunion and shortening compared with casting in a randomized trial.[223] Because of the higher prevalence of grade III open injuries of the tibia with a large proportion of blast injuries seen in military conflicts, circular external fixation has been used in several small previous series with favorable results.[224–228] A recent series of 38 patients with combat-related grade III open tibia fractures were treated with a standardized protocol including circular (Ilizarov/Taylor Spatial Frame) external fixation. Although the overall deep infection rate was 8%, exclusion of the two infections in the four patients with IIIc injuries would lower this deep infection rate to 3%.[229] In contrast, a review of tibia fractures from Operation Iraqi Freedom treated at a single institution with intramedullary nailing demonstrated an overall infection rate of 14.3%.[230]

The available literature on fixation of combat-related tibia fractures is the source of greatest debate in this review. External fixation is supported by literature at Role 2b to 3. Conversion to definitive fixation at Role 4 remains controversial. At Role 5, reamed, intramedullary nailing can be performed safely in selected patients with a lesser soft tissue injury. For grade III open injuries, circular external fixation has been shown to have lower deep infection rates.

Open fractures of the upper extremity seem to be best managed ultimately with plate fixation.[231–233] Some high-energy open fractures may benefit from a staged protocol with initial temporary external fixation.[234,235] One series of soldiers with high-energy gunshot fractures to the humerus showed a very low infection rate when managed with definitive external fixation.[236] Although functional bracing, even with war-related humerus fractures, may be favored over external fixation,[237] the current literature supports the use of temporary spanning external fixation or splint immobilization placed at Role 1–3 and transition to open plate and screw osteosynthesis for some open humerus and forearm fractures after soft tissue stabilization and closure.

Negative Pressure Wound Therapy

Wound coverage with NPWT (e.g., the VAC [KCL, San Antonio, TX]) has become standard of care in most military and civilian medical facilities. A review of the use of NPWT in the military was performed revealing overall success with the implementation of the device.[238] The use of NPWT in the combat zone appears effective, but the studies are limited by a lack of adequate control arms for comparison.[1,136] Studies have shown the device is feasible for intercontinental aeromedical evacuation without excess wound complications.[239,240] A retrospective study of combat-related injuries that assessed the role of NPWT in comparison with antimicrobial bead pouch therapy revealed that those with NPWT had more late MRSA infections, more unanticipated returns to the operating room, and overall more surgeries until closure.[126] The higher rate of *S. aureus* recovery has been previously shown in animal and human studies.[241,242] This finding of better clearance of *P. aeruginosa* in a wound versus *S. aurues* might be due to virulence of the pathogen or host factors.[242]

A randomized prospective study showed that of 58 patients with 62 open fractures those receiving NPWT had fewer infections (5.4%) compared with those not receiving NPWT (28%) ($p = 0.024$).[243] Another civilian prospective randomized study evaluating the use of NPWT in 20 calcaneous fractures, 4 pilon fractures, and 20 tibial plateau fractures found no infectious differences between NPWT and standard wound care.[244] Of note, the use of NPWT should not be employed as a substitute, or delaying method, for wound flaps, as higher rates of infections occur with delaying use of wound flaps.[245]

In an animal model, it appears that silver-impregnated gauze with the NPWT system was associated with greater reduction in bacterial load for *P. aeruginosa*, and to a greater degree, *S. aureus* than standard gauze.[246] However, wound tissue did not appear normal with this combination, raising concern that use of this product might result in surgeons

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suspecting infection even when there is no infection present. Antimicrobial beads have been assessed with and without NPWT in an animal model of *S. aureus* infection.[134] Although the NPWT and antimicrobial beads were associated with substantially more bacterial growth than antimicrobial beads alone, there was still activity in the wounds indicating that in certain situations, such as with possible issues with power loss to the suction apparatus, antimicrobial beads, and NPWT might be used effectively in combination (Joseph C. Wenke, personal communication). This study indicated that antimicrobial beads with NPWT were better than NPWT alone. It appeared that instillation of an antispetic in a NPWT system was more effective than NPWT alone or with saline solution alone; however, there was decreased tissue viability with the antiseptic (Joseph C. Wenke, personal communication). Instillation of saline in conjunction of NPWT did not demonstrate a benefit over NPWT alone in a complex orthopedic injury goat model using *P. aureuginosa*. Instillation of an antiseptic, hypochlorous acid solution, did reduce the bacteria within the wound in comparison with NPWT alone, NPWT with saline instillation, or NPWT with polyhexanide and surfactant. Clinical impression of the wounds treated with instillation of the antiseptic solutions was that they had a less healthy appearance in terms of color and consistency and the subjective impression that a greater amount of nonviable tissue was debrided from these wounds at each interval. Overall, antispectic has not been widely assessed clinically, and data discouraged hypochlorous acid (Dakin's) solution use during World War I.[247] The use of NPWT with Dakin's solution instillation (i.e., Dakin's 0.025% with the NPWT set at 125 mm Hg with instillation every 2 hours for 30 seconds with dwell time of 5 minutes) has been recently implemented for injuries that primarily occur in the lush vegetative areas of Afghanistan in patients with high bilateral lower extremity injuries, often with perineal involvement that are noted to have higher rates of invasive fungal wound infections. These severely injured patients typically require massive blood volume support and are associated with injury patterns that are not amenable to very aggressive debridement during initial or follow-up surgical management. This strategy appears to be effective but needs to be systematically analyzed to determine the unique patient populations this strategy might best be applied.

Role of Oxygen Therapy

The role of hyperbaric oxygen (HBO) has been evaluated and pursued in previous wars, especially as a potential therapy for gas gangrene.[248–250] A war extremity injury review, from 1991 to 1995, which included 388 combat-related grade III fractures, described the impact of HBO (99 provided HBO and 289 without HBO) on wound healing and infectious complications.[251] Overall, the infectious complications were less when patient management included HBO. However, this effect was substantially more common among those not receiving standard wound management and antimicrobials recommended by NATO, and there were increased cases of osteomyelitis in the HBO-treated group. Systematic reviews of HBO therapy for acute surgical and traumatic wounds revealed a lack of high-quality, valid research.[252,253]

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In addition to the role that hyperbaric oxygen therapy may or may not have on wound infection and/or prevention, there is ongoing concern regarding what effect low oxygenation might have on wounds during aeromedical evacuation of injured personnel from the combat zone to Germany and from Germany to the United States. A complex soft tissue injury in a goat model using *P. aeruginosa* contamination revealed that animals taken to pressures equivalent to an elevation of 8,800 feet for 7 hours became mildly hypoxic (O_2 saturation of 88–92%) and their wounds had more bacterial growth than controls at ground level (Warren Dorlac, personal communication). Animals provided supplemental oxygen (to increase their oxygenation saturation to >94%) were found to have with no difference in bacterial growth compared with controls at ground level. There are prospective studies that have shown mixed efficacy in preventing infectious complications with the use of higher concentrations of oxygen concentration delivery for abdominal and pelvic surgeries, although these were not associated with elevation-induced hypoxia.[254–256] Studies of the efficacy of higher oxygen concentration delivery in orthopedic trauma injuries have not been performed.

UNRESOLVED ISSUES/POTENTIAL FUTURE RESEARCH TOPICS

Role of Fungal Infections

There have been reports from the British military that casualties in the lush vegetative area of Helmand Province in Afghanistan on dismounted patrols with severe bilateral high lower extremity injuries, typically due to blast injuries and necessitating the use of tourniquets and large blood volume resuscitation, have a higher rate of invasive fungal wound infections, chiefly due to fungi belonging to the order Mucorales.[257] In civilian trauma, a study of severe extremities injuries on farms also revealed a high rate of fungi recovered from wounds; however, the nature of the injuries described in this patient population varies from the typical blast injury seen in Afghanistan.[258] Another study comparing timing of wound closure and antimicrobials performed quantitative cultures that revealed the presence of *Aspergillus* spp., *Mucor* spp., and other fungus at the time of initial wound management; although no subsequent infections secondary to these pathogens occurred.[259,260] The role of early wound evaluation with fungal cultures, fluorescent (Calcofluor) staining, and fresh frozen and traditional histopathology looking for invasive fungal infections has not been determined. In addition, the role of early empiric antifungal therapy is not known at this time for trauma-associated wound colonization with fungi.[261] There are data indicating that activity of local antifungal delivery with amphotericin B loaded beads is adequate for fungal treatment.[262] Dakin's solution (sodium hypochlorite) appears to have some activity against *Aspergillus*, but no studies assessing its activity against the Mucorales have been reported.[263] In addition, soft-tissue toxicity associated with this agent has been described.[264,265] Case series and case-control studies are underway to better characterize these infections and to better define risk factors, diagnostic strategies, and therapies.

The Role of Inflammatory Markers to Predict Infection

During the Vietnam War, there were preliminary data indicating that elevation of creatinine phosphokinase (CK), in contrast to lactic dehydrogenase (LDH) and serum glutamic oxaloacetic transaminase (SGOT), was associated with wound infections.[266] An evaluation of cytokines potentially associated with sepsis (from Belgrade, Serbia and Montenegro during 1999) revealed that IL-8, TNF-a, and IL-10 most specifically correlated with the diagnosis of combined trauma and sepsis.[267] During the current wars in Iraq and Afghanistan, a number of studies have been undertaken to evaluate various wound markers and their role with wound healing and infections. Markers that have shown an association with wound dehiscence include procalcitonin in the serum, along with increased procalcitonin, decreased RANTES protein, and decreased IL-13 concentrations in wound effluent.[268,269] Elevated metalloproteinase (MMP)-2 and MMP-7 serum levels and reduced levels of effluent MMP-3 were seen in wounds with impaired healing.[270] In addition, there has been the recovery of multipotent progenitor cells from war wound muscle tissue that might have a role in tissue engineering, and other markers of inflammation have been assessed.[271,272] Continued work needs to be undertaken in this area.

Role of Biofilms in Combat-Related Extremity Injuries

Although the role of biofilms in chronic infections is becoming more accepted, there are no data to date as to the role of biofilms in combat-related extremity injuries.[273] Although numerous investigators are assessing the ability of bacteria infecting combat-related extremity injury wounds to form biofilms in vitro, and evaluating potential therapies to prevent or disrupt these, clinical studies of the impact of biofilms are still needed.

Novel Antimicrobials and Pathogen Identification

At this time, there are inadequate antimicrobials active against MDR gram-negative pathogens in the pharmaceutical pipeline, necessitating renewed emphasis in this area. The current pathogen and antimicrobial resistant diagnostic platforms rely on old technology that typically provides a relevant clinical answer for management decision in 48 hours to 72 hours. This relegates most therapy to empiricism possibly resulting in excess antimicrobial resistance. Improvements in pathogen detection and resistance determination are necessary at this time.

CONCLUSION

Extremities are the most common injury pattern during the wars in Iraq and Afghanistan with an overall high infection rate. Continued improvement in wound care is necessary to mitigate any excess short- and long-term complications. Focus on antimicrobials, wound debridement and irrigation, and surgical interventions using the current evidence-based medicine recommendations should attempt to improve outcomes, but ongoing surveillance is necessary. In addition, continued focus on unresolved issues and future areas of research are needed to improve combat casualty care.

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RESEARCH ARTICLE

Microbial interactions and differential protein expression in *Staphylococcus aureus–Candida albicans* dual-species biofilms

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Abstract

The fungal species *Candida albicans* and the bacterial species *Staphylococcus aureus* are responsible for a majority of hospital-acquired infections and often coinfect critically ill patients as complicating polymicrobial biofilms. To investigate biofilm structure during polymicrobial growth, dual-species biofilms were imaged with confocal scanning laser microscopy. Analyses revealed a unique biofilm architecture where *S. aureus* commonly associated with the hyphal elements of *C. albicans*. This physical interaction may provide staphylococci with an invasion strategy because candidal hyphae can penetrate through epithelial layers. To further understand the molecular mechanisms possibly responsible for previously demonstrated amplified virulence during coinfection, protein expression studies were undertaken. Differential in-gel electrophoresis identified a total of 27 proteins to be significantly differentially produced by these organisms during coculture biofilm growth. Among the upregulated staphylococcal proteins was L-lactate dehydrogenase 1, which confers resistance to host-derived oxidative stressors. Among the downregulated proteins was the global transcriptional repressor of virulence factors, CodY. These findings demonstrate that the hyphae-mediated enhanced pathogenesis of *S. aureus* may not only be due to physical interactions but can also be attributed to the differential regulation of specific virulence factors induced during polymicrobial growth. Further characterization of the intricate interaction between these pathogens at the molecular level is warranted, as it may aid in the design of novel therapeutic strategies aimed at combating fungal–bacterial polymicrobial infection.

Introduction

In nature, most microorganisms are associated with surfaces in multispecies biofilm consortia. A biofilm can be defined as a community of microorganisms embedded in a self-derived polymeric matrix, attached to a surface. In a polymicrobial biofilm where multiple microbial species are closely associated, mutually beneficial interactions may develop. Polymicrobial biofilms are found in nearly every niche in the human body; the oral cavity and gastrointestinal and urogenital tracts exhibit tremendous microbial phylogenetic diversity (Aas *et al.*, 2005; Manson *et al.*, 2008). Although recent decades have witnessed a surge in the area of biofilm research, relatively little is known about the behavior of communities of mixed microorganisms, particularly fungal–bacterial biofilms. Biofilm-embedded organisms demonstrate a uniquely altered gene expression, and studies have suggested that amplified pathogenic phenotypes may emerge during multispecies interactions (Mastropaolo *et al.*, 2005; O'Connell *et al.*, 2006). One

particular biofilm-mediated microbial association, of medical interest, is that which exists between the prokaryotic pathogen *Staphylococcus aureus* and the eukaryotic pathogen *Candida albicans* (for a review, see Shirtliff *et al.*, 2009).

Methicillin-resistant *S. aureus* (MRSA) is a gram-positive coccoid bacterium that is responsible for a significant and increasing number of hospital- and community-acquired infections worldwide (Klevens *et al.*, 2007). This species possesses a number of virulence factors including adhesins, immunoavoidance factors, toxins, coagulase, and a variety of antimicrobial resistance genes (Gordon & Lowy, 2008). The multiple virulence factors of MRSA, coupled with its inherent ability to resist antibiotic therapy via antibiotic resistance gene expression and biofilm formation, have made this pathogen a significant burden to the medical community (Goetghebeur *et al.*, 2007).

Candida albicans, a fungal species commonly colonizing human mucosal surfaces, has long been adapted to the human host. However, under conditions of immune dysfunction, *C. albicans* strains cause recurrent mucosal infections and life-threatening disseminated infections (de Repentigny *et al.*, 2004). Multiple antifungal-resistant forms of *C. albicans* are also being increasingly encountered in the hospital setting (Ramage *et al.*, 2002). As a polymorphic species, *C. albicans* is capable of switching morphology between yeast, hyphal, and pseudohyphal forms, a transition central to its pathogenesis. Once in the hyphal form, host epithelial layers can be pierced, a crucial step in the initiation of candidiasis (Sudbery *et al.*, 2004).

Currently, *S. aureus* and *Candida* spp. are ranked among the top three bloodstream pathogens causing severe morbidity and mortality in hospitalized patients. Not only are *C. albicans* and *S. aureus* responsible for a substantial number of infections independently, there is increasing evidence suggesting that they are commonly associated as coinfecting organisms (Abe *et al.*, 2001; Baena-Monroy *et al.*, 2005). The clinical outcomes of polymicrobial sepsis compared with monomicrobial sepsis are grave, with significantly higher mortality rates (Pulimood *et al.*, 2002). A study by Klotz *et al.* (2007) examining the incidence of candidal bloodstream infections in hospitals reported an *S. aureus–Candida* spp. co-culture rate of up to 20%.

Candida albicans and *S. aureus* have also been coisolated from various mucosal surfaces including vaginal and oral mucosa in a biofilm mode of growth. Although *S. aureus* was thought to be a transient member of the oral microbial communities, increasing evidence from several culturing surveys suggests that it is a common isolate from the oral cavity in healthy children and adults, especially in saliva, supragingival plaque, and on the tongue (Miyake *et al.*, 1991; Smith *et al.*, 2003; Ohara-Nemoto *et al.*, 2008). More seriously, these pathogens have been coassociated with a number of polymicrobial diseases including ventilator-associated pneumonia, cystic fibrosis, superinfection of burn wounds, urinary tract infections, and denture stomatitis (Ekwempu *et al.*, 1981; Dahlen *et al.*, 1982; Siegman-Igra *et al.*, 1988; Smith *et al.*, 2003; Valenza *et al.*, 2008). Some of the most compelling evidence for this particular bacterial–fungal interaction was demonstrated through a series of studies by Carlson and colleagues (Carlson, 1983; Carlson & Johnson, 1985). The findings from these studies demonstrated a 6–70 000-fold decrease in the lethal dose 50% of *S. aureus* when coinoculated intraperitoneally with *C. albicans* in mice compared with single-species infections. Despite the significance of these observations, limited studies have examined the interactions of *C. albicans* and *S. aureus* during biofilm development, their most common infectious mode of growth.

In this study, we elucidated the nature and spatial relationship of the interactions between these two diverse pathogenic species using confocal scanning laser microscopy (CSLM) as they coexist and interact during polymicrobial biofilm growth. We have also characterized proteomic changes specific to polymicrobial culture of this cross-kingdom biofilm using two-dimensional differential in-gel electrophoresis (DIGE) and identified differentially regulated metabolic, stress, and virulence proteins via matrix-assisted laser desorption/ionization time-of-flight/time-of-flight tandem MS (MALDI-ToF/ToF MS) analysis.

Materials and methods

Strains and growth conditions

The MRSA hospital-acquired clinical isolate used in all the experiments was obtained from a patient with a biofilm-mediated infection at the University of Texas Medical Branch – Galveston and previously designated as strain M2 (Brady *et al.*, 2006). The well-characterized *C. albicans* lab strain SC5314 was used for all the experiments (Gillum *et al.*, 1984). In addition, *S. aureus* strain Seattle 1945 [containing a plasmid encoding for chloramphenicol resistance and green fluorescent protein (GFP) expression under control of the *sarA* promoter] and the constitutively GFP-expressing *C. albicans* strain CAF2-1 were also used (Morschhauser *et al.*, 1998; Leid *et al.*, 2002). The following bacterial strains were also used: *Staphylococcus epidermidis* (clinical isolate), *Pseudomonas aeruginosa* (PA01), *Streptococcus pyogenes* (clinical isolate), *Bacillus subtilis* (ATCC #6633), and a laboratory strain of *Escherichia coli* (DH5-α).

For all studies, an aliquot of a glycerol stock of *C. albicans* strain SC5314 or GFP-expressing CAF2-1 was grown and maintained on Sabouraud dextrose agar (BBL, Cockeysville, MD). Cultures were grown overnight in yeast peptone dextrose (YPD) (BBL, Sparks, MD) in an orbital shaker (120 r.p.m.) at 37 °C under aerobic conditions. Yeast cells were harvested and washed twice in sterile phosphate-

buffered saline (PBS). Starter cultures of clinical isolates of *S. aureus* (M2), GFP-expressing *S. aureus* (Seattle 1945), *S. epidermidis* (clinical isolate), *P. aeruginosa* (PA01), *S. pyogenes* (clinical isolate), *B. subtilis* (ATCC #6633), and a laboratory strain of *E. coli* (DH5-α) were grown in trypticase soy broth (TSB) (Remel, Lenexa, KS) and incubated overnight at 37 °C. Fresh log-phase bacterial starter cultures were grown by diluting the overnight culture 1 : 100 in fresh TSB for 3 h. Bacterial cultures were then washed twice in sterile PBS. Dual-species biofilms were grown in RPMI 1640 buffered with HEPES and supplemented with L-glutamine (Invitrogen, Grand Island, NY) and 5% heat-inactivated fetal bovine serum (RPMI–FBS) (Hyclone, Logan, UT) or YPD containing 5% FBS medium (YPD–FBS).

Biofilm growth

Staphylococcus aureus was grown as noted above and diluted to an $OD_{600\,nm}$ of 0.1. *Candida albicans* overnight cultures were grown as described above and diluted to an $OD_{540\,nm}$ of 1.0. Biofilms for protein nucleic acid (PNA)-FISH were grown for 24 h on glass coverslips in polystyrene 6-well plates (Corning, Lowell, MA) in 5 mL of RPMI–FBS. Dual-species biofilms were grown by inoculating wells with 50 μL of both species suspensions. PNA-FISH was performed as per the manufacturer's protocol (Advandx, Woburn, MA) with a Cy3-labeled *C. albicans*/fluorescein isothiocyanate (FITC)-labeled *S. aureus* PNA probe cocktail. Nonadherent cells were removed by washing with PBS before imaging. Fluorescence was captured with a Zeiss LSM 510 (Carl Zeiss, Thornwood, NY) confocal microscope using a ×20 objective and a FITC/Texas Red dual-band filter. In order to confirm the strain-independent interaction of *S. aureus* and *C. albicans*, dual-species biofilms of GFP-expressing strains were grown on glass coverslips in RPMI–FBS supplemented with 10 μg mL^{-1} chloramphenicol. Coverslips were processed for microscopy as described above. Finally, microbial protein samples for proteomic studies were prepared by growing mono- or dual-species biofilms in 6-well polystyrene plates as above in either 5 mL of RPMI–FBS (for experiments with hyphae) or YPD–FBS (for experiments with yeast cells) at 37 °C for 24 h.

Hyphal–bacterial attachment assay

Hyphae formation was induced by first growing *C. albicans* as described previously on glass coverslips in 6-well plates in 3 mL RPMI–FBS for 4 h. Nonadherent hyphae were removed by gently washing the coverslips in PBS, followed by the addition of 3 mL of fresh RPMI–FBS. Log-phase bacterial cell suspensions were washed in PBS, equalized to an $OD_{600\,nm}$ of 0.1, and added to the *C. albicans* biofilms. Plates were placed on a rotary shaker to distribute the bacteria evenly and incubated for 1 h at 37 °C. Following incubation, nonadherent cells were removed by gently washing the coverslips in PBS and then examined using phase-contrast microscopy under a ×100 oil-immersion objective. The total number of bacterial cells per field and attached bacteria per hyphae were counted. Percent attachment was calculated by dividing the number of attached bacteria by the total number of bacteria. A total of 10 random fields per coverslip were analyzed.

Morphological specificity binding assay

Hyphal and blastospore biofilms were grown as described above in RPMI–FBS or YPD–FBS, respectively. Nonadherent cells were gently removed by washing in PBS. Log-phase staphylococcal cell suspensions were added to the *C. albicans* biofilms, shaken, and incubated for 1 h 37 °C. Following incubation, nonadherent cells were removed by gently washing the coverslips in PBS and then examined using phase-contrast microscopy under a ×100 oil-immersion objective. Attachment rates were calculated by counting the total number of yeast cells or hyphae per field as well as the number of attached *S. aureus* cells. These numbers were divided to calculate the average number of *S. aureus* attached per *C. albicans* cell. A total of 10 random fields per coverslip were analyzed.

Microbial viability assay

Polymicrobial biofilms were grown on glass coverslips as described previously using *C. albicans* SC5314 and *S. aureus* M2. Coverslips were removed from the incubator after 12, 24, and 40 h of growth. Biofilms were washed briefly in PBS, placed into sterile 6-well plates, and stained using the BacLight LIVE/DEAD viability kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The BacLight LIVE/DEAD system stains live cells green (Syto9), while dead cells appear red (propidium iodide). Coverslips were then mounted onto glass slides with Vectashield (Vector Laboratories, Burlingame, CA) and processed for CSLM. The spatial arrangement of the polymicrobial biofilm was determined by analysis of confocal *z*-axis image slices using the LSMIX software package (Carl Zeiss).

Proteomic analysis

Plates containing 24-h biofilms were gently shaken on a rotary shaker for 1 min and then the culture supernatants were discarded. To remove the biofilms from the wells, 1 mL of cell wash buffer (10 mM Tris, 5 mM Mg acetate, pH 8.0) supplemented with 3 mM phenylmethanesulfonyl fluoride was added and a cell culture tissue scraper was used to remove attached cells. Cells were then washed twice in cell wash buffer, resuspended in 1 mL lysis buffer (30 mM Tris, 4 M urea, 2 M thiourea, 1% CHAPS), and incubated on ice

for 10 min. Cells were then mechanically disrupted in a FastPrep FP120 (ThermoSavant, Holbrook, NY) using 0.1 mm zirconia beads (Biospec Products, Bartlesville, OK) for 30 s, followed by a 2-min incubation on ice; the process was repeated for a total of 10 times. Suspensions were centrifuged for 10 min at 14 000 ***g*** and supernatants were removed and protein was quantified spectrophotometrically using the Advanced Protein Assay Reagent #2 (Cytoskeleton Inc., Denver, CO). Crude protein extracts were precipitated and purified with Perfect-Focus reagent as per the manufacturer's directions (G-Biosciences, Maryland Heights, MO) and stored at $-70\,^{\circ}C$ until used.

Two-dimensional DIGE was performed according to the concepts of O'Farrell and Minden and outlined by Sauer and Camper (O'Farrell, 1975; Sauer & Camper, 2001; Minden, 2007). Protein labeling was performed using the DIGE system (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. To achieve sufficient protein rehydration, 100 μg of each protein sample was resuspended in 150 μL of rehydration buffer (30 mM Tris, 7 M urea, 2 M thiourea, 2.5% CHAPS). Following rehydration, the pH was adjusted to 8.5 with dilute NaOH or HCl as needed. *Candida albicans* proteins were labeled with Cy2, *S. aureus* proteins were labeled with Cy3, and co-cultures were labeled with Cy5 at a ratio of 2 pmol CyDye μg^{-1} protein. Samples were incubated for 30 min on ice and kept protected from light. Following CyDye labeling, 15 μL of 10 mM lysine was added for 10 min to quench excess CyDye. Samples were combined and a final concentration of 35 mM DTT and 1.6% Pharmalyte 3-10 was added. Samples were applied to 24 cm, pH 3–10 (linear) Immobiline Dry-Strips (IPG) (GE Healthcare). Proteins were separated in the first dimension by their isoelectric point using a Multiphor II (Amersham) as per the manufacturer's directions. Before the second dimension, IPG strips were equilibrated and applied to 12% 26 cm × 20 cm sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Protein spots were resolved in the second dimension using a Höefer DALT Vertical System and fluorescence was captured using the Typhoon Imager 9400 (GE Healthcare). Following fluorescence scanning, gels were nondestructively silver stained for spot excision (Gharahdaghi *et al.*, 1999). Protein spots that were upregulated in six out of six gels were selected for MALDI-ToF/ToF MS analysis as described previously (Brady *et al.*, 2006).

Statistics

All studies were performed in triplicate at a minimum. In addition, all cell enumerations were performed on a minimum of 10 fields of view and at least 400 cells. A Student's *t*-test was used to compare microbial numbers, with a $P < 0.05$ representing a statistical significance.

Results

Hyphal–bacterial attachment assay

In order to assess the potential for hyphal–bacterial interactions, we tested a panel of various bacterial species displaying a wide variety of phenotypes including cell morphology, motility, ecological niche, and Gram stain identity for hyphal interaction. *Candida albicans* biofilms were grown overnight on glass coverslips, washed, and various bacterial strains added for 1 h. Hyphal binding was measured via phase-contrast microscopy as the number of attached bacterial cells to *C. albicans* hyphae divided by the total number of bacterial cells per microscopic field and reported as a percentage (Fig. 1). Percent counts demonstrated that *S. aureus* had the highest hyphal association (56%), followed by *S. pyogenes* and *S. epidermidis* (25%). *Pseudomonas aeruginosa*, a gram-negative motile rod and known hyphae binder, had a hyphal association of (17%), while *E. coli*, also a gram-negative rod, and *B. subtilis*, a gram-positive bacillus, demonstrated the lowest hyphal binding (5.7% and 2.5%, respectively).

PNA-FISH

Because of the strong hyphal binding exhibited, fluorescence microscopy using species-specific PNA-FISH probes was used to visualize the physical interaction between *C. albicans*

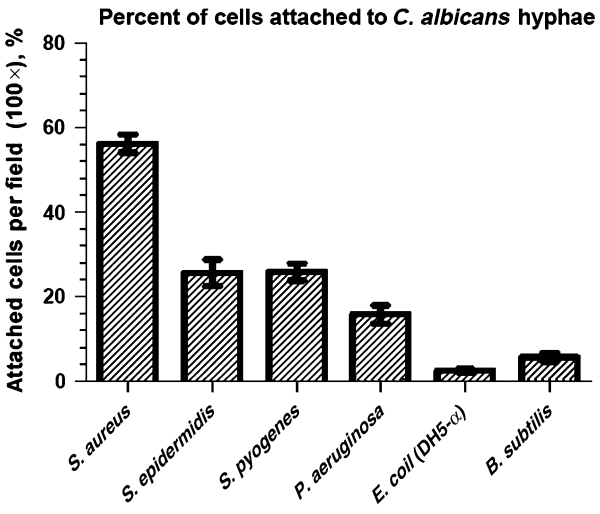

Fig. 1. Bacterial attachment assay. *Candida albicans* biofilms were grown for 3 h in RPMI to induce hyphae formation and incubated for 1 h with the following bacteria: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli* (DH5-α). Nonadherent cells were removed by washing and the remaining cells were counted by phase-contrast microscopy. Percent hyphal attachment was assessed by counting the number of bacteria associated with the hyphae divided by the number of total bacteria per field. Ten fields were chosen at random and averaged; the experiment was repeated in triplicate. Error bars represent SD.

and *S. aureus* in an *in vitro* dual-species biofilm. Images revealed extensive adherence of *S. aureus* to *C. albicans*, with a preferential association to the invasive hyphal elements of *C. albicans* (Fig. 2a and c). In areas of dense hyphal biofilm growth, *S. aureus* could be seen completely covering *C. albicans* (Fig. 2b). To show the specificity of *S. aureus* for binding the hyphal form of *C. albicans*, polymicrobial interactions were assessed using both hyphae and yeast biofilms. Quantitative counts demonstrated a 30-fold increase in *S. aureus* binding to hyphae as compared with *C. albicans* yeast cells. These observations were confirmed by similar experiments performed using different GFP-expressing strains of *C. albicans* and *S. aureus* where a similar adherence pattern was demonstrated, confirming that this interaction is strain independent (Fig. 2e and f).

Microbial viability assay

The BacLight LIVE/DEAD cell viability assay was used to determine whether fungal or bacterial cells were killed during polymicrobial biofilm growth and to assess the spatial arrangement of the biofilm. After 16, 24, and 40 h of growth, both cell types were viable as visualized by green fluorescent staining (Syto9) with an apparent lack of red fluorescence (propidium iodide) (Fig. 3a). In addition to staining for cell viability, the spatial arrangement of the dual-species biofilm was characterized by confocal *z*-stack imaging analysis. Bottom, middle, and top representative *z*-axis image slices from a 24-h polymicrobial biofilm show the presence of *S. aureus* attached to the hyphae of *C. albicans* throughout the entire biofilm architecture (Fig. 3b).

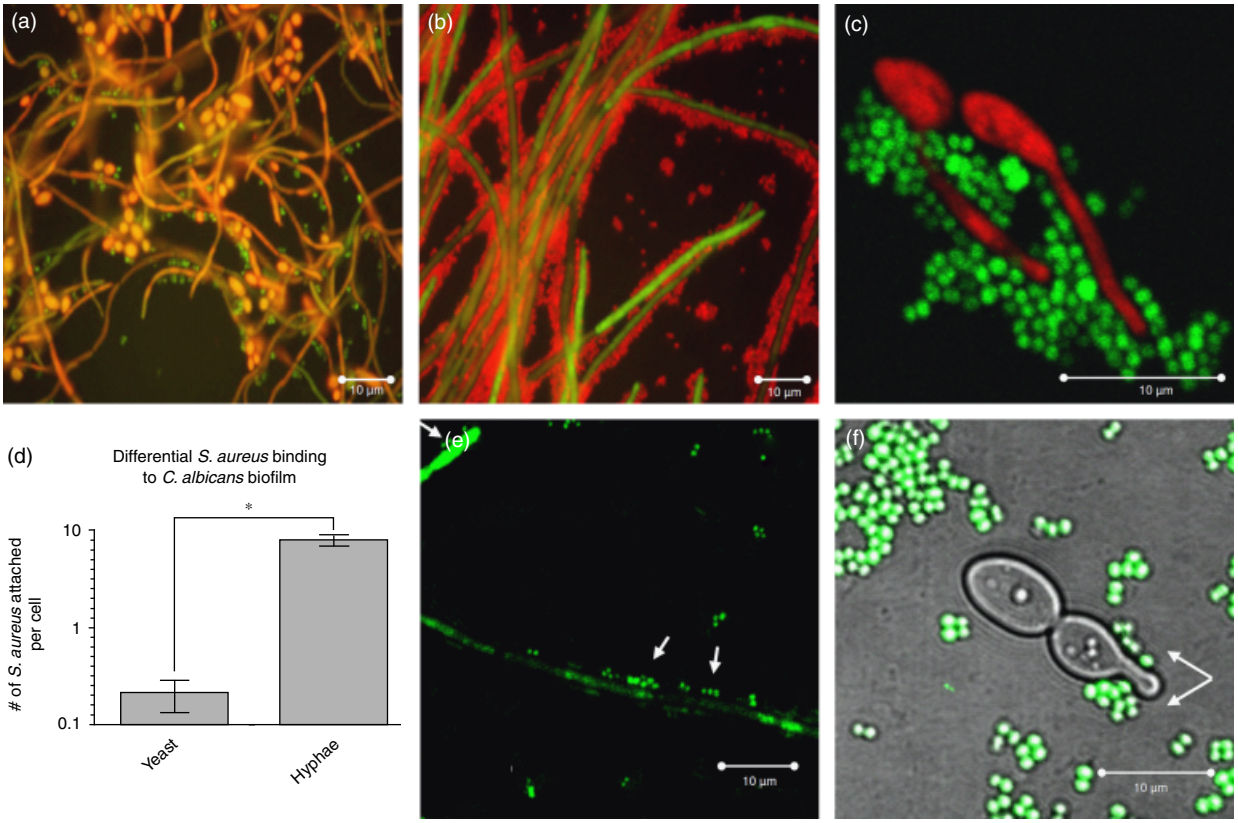

Fig. 2. Biofilm architecture of *Candida albicans* and *Staphylococcus aureus* 24-h dual-species biofilm using PNA-FISH and GFP-expressing microorganisms. (a) *Staphylococcus aureus* (FITC-labeled probe, green) has a greater tropism for the hyphal form of *C. albicans* (TAMRA-labeled probe, red) compared with the yeast form. Field of view diameter is 150 μm. (b) An area of *C. albicans* (FITC-labeled probe, green) hyphal biofilm growth is completely covered by *S. aureus* (Cy3-labeled probe, red). (c) A × 63 zoom image showing staphylococci (FITC-labeled probe, green) binding to only the hyphal filaments of *C. albicans* (Cy3-conjugated probe, red). (d) Graph representing the average number of *S. aureus* cells attached per *C. albicans* cell during polymicrobial biofilm growth. Ten fields were chosen at random for counting and the experiment was repeated in triplicate. Error bars represent the SD. (e) *Staphylococcus aureus* (white arrows), expressing GFP under control of the *sarA* promoter, was found to be associated to GFP-expressing *C. albicans* hyphae. (f) *Staphylococcus aureus* (white arrows) demonstrating preferential binding to a *C. albicans* germ tube without binding to the yeast cell. Fluorescence was captured with a × 63 oil-immersion objective and FITC/DICIII, FITC/Texas Red filter sets. Asterisk (*) denotes a statistically significant difference at $P < 0.05$.

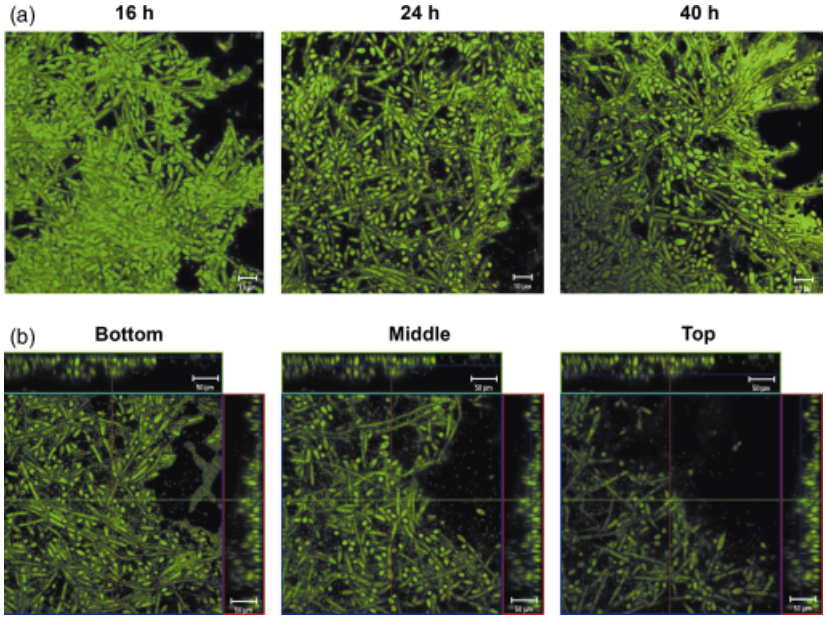

Fig. 3. Viability and spatial arrangement in the dual-species biofilm. *Candida albicans–Staphylococcus aureus* biofilms were grown for various time points on glass coverslips, stained with BacLight LIVE/DEAD, and processed for CSLM. (a) At all the time points tested, both bacteria and fungi appear healthy as measured by the presence of green fluorescence (Syto9) and the absence of red (propidium iodide). (b) Representative confocal *z*-stack images of a typical 24-h dual-species biofilm demonstrating the presence of *S. aureus* attached to *C. albicans* hyphae throughout the bottom, middle, and top layers.

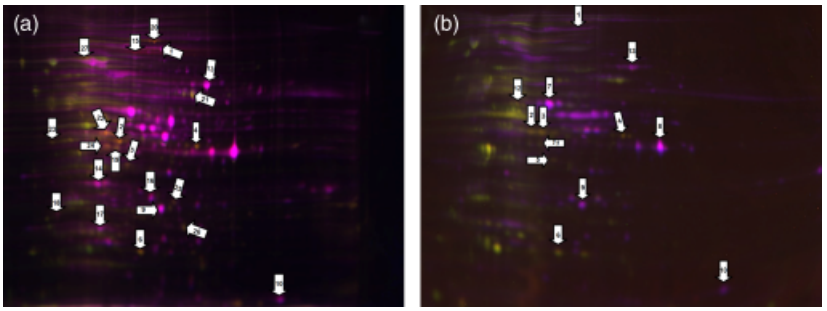

Fig. 4. Representative DIGE gel from mono- and dual-species biofilms. Whole-cell lysates, enriched in the cytoplasmic fraction, were obtained from 24-h biofilms. Proteins (100 μg) were differentially labeled with CyDye: *Candida albicans* labeled with Cy2 (blue), *Staphylococcus aureus* labeled with Cy3 (green), dual-species biofilm proteins labeled with Cy5 (red). Proteins were focused in the first dimension on pH 3–10 IEF strips and resolved in the second dimension on 12.5% polyacrylamide gels. (a) Representative gel from staphylococcal–yeast biofilms. (b) Representative gel from staphylococcal–hyphal biofilms.

Proteomic analysis

In order to identify other factors that may lead to increased virulence during coinfection, unfractionated, whole-cell proteins from 24 h *in vitro* biofilms were harvested, purified, and differentially lysine-labeled with NHS-ester CyDyes. Labeled proteins were then combined and subjected to isoelectric focusing and second dimension analysis. Representative gels from either mono- or dual-species biofilms composed of *S. aureus* and *C. albicans* yeast cells (Fig. 4a) or *S. aureus* and *C. albicans* hyphal cells (Fig. 4b) are shown. Spots were considered for MALDI-ToF/ToF MS identification if they were reproducible on six out of six gels. In this global proteomics screen, we identified 27 proteins that were upregulated in the co-culture biofilm. Among these were proteins important for growth and metabolism and others of hypothetical function. Most notable of interest were those proteins implicated in microbial stress and enhanced virulence of both species (Table 1).

Discussion

Previous studies have identified that *C. albicans–S. aureus* intraperitoneal coinfections resulted in enhanced virulence and lethality in a mouse model, but a detailed description of the polymicrobial interactions between these pathogens has remained undefined (Carlson, 1983; Carlson & Johnson, 1985). To this end, this study was designed to examine the physical interactions and the differential protein expression

Table 1. Proteins upregulated in the dual-species biofilm

Spot	MW (Da)	pI	Organism	Identity	Protein name	Peptide matches	Protein score confidence interval (%)	Accession number	Function
(A) Proteins upregulated in staphylococcal–yeast biofilms									
1A	84624.8	5.96	*C. albicans*	Putative mitochondrial aconitate hydratase	Aco1p	19	100	68479387	Carbohydrate metabolism; tricarboxylic acid cycle
14A	21481.5	5.15	*C. albicans*	Similar to heat shock protein 5	Similar to Hsp5	9	100	68469633	Cellular stress response; protein folding
15A	91795.2	6.35	*C. albicans*	Heat shock protein 78	Hsp78p	16	100	31076745	Cellular stress response; protein folding
16A	26893.9	5.74	*C. albicans*	Triosephosphate isomerase	Tpi1p	10	100	7270988	Glycolysis; gluconeogenesis; fatty acid biosynthesis
17A	21960.3	4.98	*C. albicans*	Thioredoxin peroxidase	Tsa1p	7	99.99	68479826	Cellular stress response; antioxidant
18A	49188.7	7.36	*C. albicans*	Metal-binding activator 1	Mac1p	6	85.35	68471167	Copper-binding transcriptional regulator; cellular stress response
19A	95340.7	5.68	*S. aureus*	Alcohol dehydrogenase, iron containing	Adh	15	100	57651152	Carbon utilization; alcohol metabolism
20A	95397.8	5.73	*S. aureus*	Putative aldehyde-alcohol dehydrogenase	AdhE	16	100	49482391	Carbon utilization; putative peroxide scavenger
21A	56138.6	6.02	*S. aureus*	Probable malate:quinone oxidoreductase	Mqo1	21	100	82752186	Carbohydrate metabolism; tricarboxylic acid cycle
22A	37820.9	5.14	*S. aureus*	Ornithine carbamoyltransferase	ArgF	12	100	49484831	Amino acid biosynthesis
23A	35194.1	4.65	*S. aureus*	Pyruvate dehydrogenase complex E1 component β	PdhB	11	100	57651703	Glycolysis; oxidoreductase
24A	35539.3	5.36	*S. aureus*	Carbamate kinase	ArcC1	17	100	49484829	L-Arginine degradation
25A	28737.4	5.87	*S. aureus*	Transcriptional repressor CodY	CodY	8	99.97	15924245	Decreased hemolysin, biofilm, and quorum-sensing function
26A	23092.2	6.08	*S. aureus*	Uracil phosphoribosyl transferase	Upp	10	100	15925102	Pyrimidine metabolism
27A	63331.2	5.2	*S. aureus*	Pyruvate kinase	Pyk	27	100	49483939	Carbohydrate metabolism; glycolysis
(B) Proteins upregulated in staphylococcal–hyphal biofilms									
1B	93865.5	6.07	*C. albicans*	Translation elongation factor 2	Eft2p	4	100	68481380	Protein synthesis
8B	35924.7	6.61	*C. albicans*	Glyceraldehyde 3 phosphate dehydrogenase	Thd1p	15	100	68472227	Carbohydrate metabolism; glycolysis
11B	33026.2	5.4	*S. aureus*	Cysteine synthase	CysK	4	100	82750220	Cysteine biosynthesis
3B	29543.3	5	*S. aureus*	L-Lactate dehydrogenase	Ldh1	9	100	87161566	Growth during nitrosative stress
12B	40322.9	5.2	*S. aureus*	Alanine dehydrogenase 1	Ald1	15	100	21283057	Cell wall synthesis; oxidation reduction
(C) Proteins upregulated in both biofilm conditions									
9A,B	21481.5	5.79	*C. albicans*	Similar to phosphoglycerate mutase	Gpm1p	16	100	68469783	Carbohydrate metabolism; glycolysis
10A,B	17677.9	7.74	*C. albicans*	Cyclophilin type peptidyl-prolyl *cis–trans* isomerase	Cyp1p	4	100	68469052	Protein folding; cellular stress response
13A,B	55751.8	6.54	*C. albicans*	Pyruvate kinase	Pyk1p	16	100	68482226	Carbohydrate metabolism; glycolysis
2A,B	36423.8	5.34	*S. aureus*	Alcohol dehydrogenase	Adh	12	100	21282297	Carbon utilization; alcohol metabolism
5A,B	29434.3	5.34	*S. aureus*	30s ribosomal protein S2	RpsB	6	100	57651825	Protein synthesis; stress response
6A,B	18520.5	5.6	*S. aureus*	Similar to universal stress protein family	Similar to UspA1	7	95.7	15924700	Cellular stress response
4A,B	37381.6	6.08	*S. aureus*	Threonine dehydratase	IlvA	16	100	147733998	Amino acid metabolism

occurring during *C. albicans–S. aureus* polymicrobial biofilm growth.

Because *C. albicans* bacterial binding has been reported previously, the relative *C. albicans* hyphal-binding affinity of other bacteria was evaluated and compared with that of *S. aureus* (Fig. 1). Comparative adherence assays demonstrated that all bacterial species tested, including the more closely related species of *S. pyogenes* and *S. epidermidis*, associated with the hyphae of *C. albicans* significantly less than *S. aureus*. Differences in hyphal binding between various

bacteria may be due to differences in surface protein expression or as yet unidentified microbial adhesins. Our assay also demonstrated significantly lower hyphal binding of the well-described *C. albicans*-interacting bacterial species *P. aeruginosa* compared with *S. aureus*. Even with this comparatively lower binding affinity, Hogan *et al.* (2004) have demonstrated that *P. aeruginosa* is capable of killing the hyphae of *C. albicans*, through a process involving the homoserine lactone quorum-sensing molecule, 3-oxo-C12. These observations indicate that, unlike the seemingly mutualistic *C. albicans*–*S. aureus* relationship demonstrated by our studies, the interaction between *C. albicans* and *P. aeruginosa* seems to be antagonistic.

Because of the significantly increased rates of staphylococcal–fungal association identified in the hyphal–bacterial attachment screen, polymicrobial growth was visualized by fluorescence microscopy in order to determine the architecture of the co-culture biofilm. Imaging analysis revealed *S. aureus* adhering to the invasive hyphal filaments of *C. albicans*, but not the round yeast cells (Fig. 2). Confocal *z*-stack imaging showed *S. aureus* to be distributed along the hyphal filaments throughout the entire biofilm architecture (Fig. 3b). These findings differ from the recent findings by Harriott & Noverr (2009) investigating increased drug resistance in polymicrobial biofilms in which *S. aureus* was noted to be attached to hyphal elements mostly in the uppermost layers of the biofilm. Differences in biofilm growth substratum and medium may partially account for these discrepancies. Preference for binding the hyphae of *C. albicans* has been reported in a number of other species, including *S. pyogenes*, *Acinetobacter baumannii*, and *P. aeruginosa* (Cunningham, 2000; Hogan & Kolter, 2002; Peleg *et al.*, 2008; Bamford *et al.*, 2009). Many of these previously identified *C. albicans*–bacteria interactions result in fungal and/or bacterial killing during co-culture; however, the *C. albicans*–*S. aureus* interaction described in this study appears to be nonlethal for either organism as measured by the LIVE/DEAD cell viability assay (Fig. 3a). The lack of an antagonistic relationship during polymicrobial growth may have important implications for the enhancement of virulence during coinfection and may partially explain the relatively high rate of co-culture for these organisms. Combined, these important findings highlight the diversity of the interactions that take place between these human pathogens.

In light of the observed extensive association between *S. aureus* and *C. albicans* hyphae, we hypothesized that protein expression may be modulated in the dual-species environment, which could have important implications during coinfection. Hyphal binding may result in altered virulence factor production, augmenting immunoavoidance and/or damage to the host as has been seen in other species (Richard *et al.*, 2002; Sibley *et al.*, 2008). In order to further characterize the molecular interactions between *C. albicans* and *S. aureus* and to identify the factors that may be responsible for their infectious synergism, a global proteomics approach was utilized; the upregulated proteins identified are listed in Table 1. Among the 27 differentially regulated proteins, some were upregulated either uniquely in the staphylococcal–yeast or staphylococcal–hyphae biofilms or in both co-culture conditions compared with mono-species cultures. These proteins were mainly involved in growth, metabolism, or response to stress including proteins that are inducible upon heat, oxidative, nutrient, and antibacterial stress.

Several stress-related proteins, known to be induced upon heat, oxidative, and antibacterial stress, were found to be consistently upregulated by *S. aureus*, indicating the presence of a stress response by *S. aureus* to both *C. albicans* yeast and hyphal forms (Table 1) (Kvint *et al.*, 2003). Similarly, Cyp1p, a *cis–trans* isomerase involved in protein folding and upregulated during oxidative and nutritional stress, was upregulated in *C. albicans* (Dartigalongue & Raina, 1998; Andreeva *et al.*, 1999; Wen *et al.*, 2005). The upregulation of the uspA-like protein, Cyp1p, and RpsB, a ribosomal protein, is consistent with the findings of upregulated proteins *in vivo* during *Mycobacterium avium* infection and emphasizes that these proteins may be important in resisting heat shock and stress inside the host (Hughes *et al.*, 2007).

Many growth and metabolic proteins in both *C. albicans* and *S. aureus* were upregulated in the mixed biofilm. Contrary to our expectations, however, the majority of the upregulated proteins were present in the staphylococcal–yeast biofilm. Interestingly, *C. albicans* yeast cells demonstrated the upregulation of a significant number of proteins involved in cell stress, including the heat shock proteins, which are highly inducible upon cell stresses including heat, hypoxia, UV exposure, starvation, toxin exposure, and dehydration (Table 1) (Matthews & Burnie, 1992). It is possible that staphylococcal binding to *C. albicans* blastospores within the polymicrobial biofilm may have been evolutionarily selected against under seemingly 'stressful' conditions.

In *C. albicans*, Mac1p is a transcription factor that facilitates the uptake of copper. Copper is an important cofactor for a wide variety of cellular enzymes that carry out essential biological processes such as respiration (Marvin *et al.*, 2003). Furthermore, copper is believed to play a detrimental role in protection against oxidative stress, which provides an additional explanation for the observed upregulation of Mac1p. This is corroborated by the observed aforementioned concomitant upregulation of various stress response proteins by *C. albicans* yeast cells. Combined, these findings clearly indicate that the presence of *S. aureus* induces a stress response by *C. albicans*.

Among the proteins of note found to be upregulated in *C. albicans* yeast cells was Tsa1p, a thioredoxin peroxidase

important for detoxification after peroxide stress (Urban *et al.*, 2005), and aconitate hydratase, which is highly susceptible to oxidation under stressed conditions (Tang *et al.*, 2002; Matasova & Popova, 2008). Few proteins were found to be upregulated in the staphylococcal–hyphae biofilm in either organism. In *C. albicans*, the expression of Tef2p, a GTP-binding translational elongation factor important for protein synthesis, was increased (Capa *et al.*, 1998). In *S. aureus*, there was increased expression of alanine dehydrogenase, shown to be involved in the metabolism of alanine and suggested to have a role in bacterial cell wall synthesis (Andersen *et al.*, 1992). In addition, cysteine synthase involved in the biosynthesis of cysteine was also found to be upregulated in *S. aureus*. *Staphylococcus aureus* mutants deficient in cysteine synthase are more susceptible to oxidative stress, acid, and phosphate-limiting conditions due to the role of cysteine in stress response and survival mechanisms (Lithgow *et al.*, 2004).

Staphylococcal gene products that have been previously shown to play an important role in virulence and pathogenesis were also shown to be differentially regulated under coculture conditions compared with mono-species cultures. In the staphylococcal–yeast biofilm, CodY, a transcriptional repressor of a variety of *S. aureus* virulence factors exhibited increased expression (Levdikov *et al.*, 2006). This protein has was shown to repress PIA-dependent biofilm formation, the production of hemolysins alpha and delta, and proteins involved in the global regulator of virulence, the *agr*-dependent quorum-sensing system (Frees *et al.*, 2005; Majerczyk *et al.*, 2008). However, CodY was downregulated under the staphylococcal–hyphal biofilm growth conditions. Therefore, decreased CodY expression may enable enhanced toxin-mediated virulence and increased biofilm formation in *S. aureus*.

The virulence-associated L-lactate dehydrogenase 1 (Ldh1), an enzyme involved in the generation of L-lactate during fermentation, was upregulated in the staphylococcal–hyphal biofilm, but not in the staphylococcal–yeast biofilm. Recently, biochemical studies by Richardson and colleagues demonstrated that *S. aureus* Ldh1 is uniquely inducible under nitrosative stress conditions, enabling *S. aureus* to persist in the host in the presence of host-derived nitric oxide. Furthermore, an *S. aureus ldh1* mutant exhibited attenuated virulence compared with wild-type *S. aureus* in a mouse model of systemic infection (Richardson *et al.*, 2008). Closely related staphylococcal species, *S. epidermidis* and *Staphylococcus saprophyticus*, lack Ldh1 and therefore cannot survive under conditions of nitric oxide stress as encountered in host macrophages and neutrophils.

The increased expression of CodY and downregulation of Ldh1 lead us to hypothesize that *S. aureus* may downregulate its virulence while coexisting with *C. albicans* yeast cells at a mucosal surface such as at vaginal, gastrointestinal, or oral tracts as a strategy to remain in a commensal state at these sites, thereby evading detection and clearance by the host immune system. Conversely, candidal germination appears to induce *S. aureus* virulence and biofilm formation capability through the downregulation of CodY expression. The simultaneous increase in Ldh1 expression could potentially combat nitric oxide produced by the host in response to *C. albicans* hyphal invasion (Oliveira *et al.*, 2007). While these proteomics studies are not a comprehensive analysis of the entire proteome, they do demonstrate the plasticity of global protein expression unique to polymicrobial growth. Further experiments to address these polymicrobial-enhanced immunoavoidance and virulence mechanisms, as well as the possible differential expression of cell wall proteins and secreted factors, are warranted and currently underway in our laboratories.

In conclusion, this study characterizes a unique microbial association within the context of a polymicrobial biofilm, in which *S. aureus* binds the hyphal elements of *C. albicans*. In addition, it establishes the presence of a robust and dynamic interaction between two diverse and significant human pathogens by demonstrating the upregulation of several putative virulence factors specific to polymicrobial growth. The findings generated from this investigation will contribute to our understanding of the complex and clinically significant interactions that take place between microbial species as they coexist in the host and during infectious processes. Therefore, continued epidemiologic and laboratory research is needed to better characterize and understand these pathogens in the context of complicated polymicrobial infections, allowing for improved diagnostic and therapeutic strategies in the future.

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Prevention of Infections Associated With Combat-Related Eye, Maxillofacial, and Neck Injuries

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Abstract: The percentage of combat wounds involving the eyes, maxillofacial, and neck regions reported in the literature is increasing, representing 36% of all combat-related injuries at the start of the Iraq War. Recent meta-analysis of 21st century eye, maxillofacial, and neck injuries described combat injury incidences of 8% to 20% for the face, 2% to 11% for the neck, and 0.5% to 13% for the eye and periocular structures. This article reviews recent data from military and civilian studies to support evidence-based recommendations for the prevention of infections associated with combat-related eye, maxillofacial, and neck injuries. The major emphasis of this review is on recent developments in surgical practice as new antimicrobial studies were not performed. Further studies of bacterial infection epidemiology and postinjury antimicrobial use in combat-related injuries to the eyes, maxillofacial, and neck region are needed to improve evidence-based medicine recommendations. This evidence-based medicine review was produced to support the *Guidelines for the Prevention of Infections associated with Combat-related Injuries: 2011 Update* contained in this supplement of *Journal of Trauma*.

Key Words: War, Trauma, Head, Face.

(*J Trauma.* 2011;71: S000–S000)

Combat operations in Iraq and Afghanistan have continued since guidelines were released for prophylaxis and treatment of combat-related eye, maxillofacial, and neck (EMFN) injuries in 2008.[1] Recent studies indicate that EFMN injuries comprised 36.2% of all injuries at the onset of the Iraq war,[2] although larger studies showed a slightly lower rate of 29% to 30%.[3,4] A meta-analysis of all studies from the 21st century[5]found incidence of injury to the face between 8% and 20%,[3,6–10] the neck between 2% and 11%,[3,6,7,9,10] and the eye between 0.5% and 13%.[3,7,11] Further data on eye injuries alone show approximately a 6% incidence, down from the first Gulf war rate of 13% but consistent with the Israeli defense forces experience in the 1960s and 1970s.[12] Regardless, EMFN injuries now far exceed those reported from any previous conflicts. Whether this is a consequence of changes in defensive posture (e.g., body armor deployment and use of armored transportation), shifts in enemy tactics and weaponry, or the urban battlefield remains unclear (although the urban war in Somalia experienced a 12% EMFN injury rate).[13] This article reviews recent developments in epidemiology, postinjury antimicrobials, and surgical techniques to prevent infection of EMFN injuries sustained in combat.

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EPIDEMIOLOGY/MICROBIOLOGY OF WOUND COLONIZATION/INFECTION

AQ: 1

Maxillofacial and Neck Injuries

We have previously noted that the EMFN infection wound rate from the Vietnam War was 7% to 42%.[14,15] In the Balkans, conflict wounds became infected postoperatively in 19% of war-wounded patients,[16] and in the Iran-Iraq war, 11% of maxillofacial injuries were complicated by infection.[17] Two small case series from the Iraq war of patients undergoing open reduction and internal fixation of fractures at Role 3 (e.g., combat support hospital) described a 0% infection rate among 17 patients[18]; however, a second review of 130 patients described a 24% infection rate.[19]

Actual pathogen descriptions of maxillofacial infections in combat-associated wounds are limited and include *Klebsiella* spp., and fungi (likely *Candida* spp.)[20]; *Pseudomonas* spp., *Staphylococcus aureus*, and *Escherichia coli*[20,21]; *Proteus mirabilis*, *Bacteroides fragilis*, *Peptococcus* spp., and *Peptostreptococcus* spp.[21]; and *E. coli* and *Streptococcus pyogenes*.[16] Unfortunately, infection rates are not reported so it is not clear if these reported microbes represent true infection or just colonization. Since our last review, no new studies from current conflicts have described bacterial epidemiology of infection following maxillofacial trauma. One study described a 7-year retrospective review of 38 patients with facial gunshot wounds, reporting a 10.5% infection rate, but pathogens were not described nor were locations or causes of infection or how they were treated.[22] Postinjury antimicrobials with broad activity against the 12 previously described pathogens to prevent perioperative infections might

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TABLE 1. Suggested Antimicrobials and Duration of Administration for Postinjury Use in Maxillofacial and Neck Combat-Related Injuries

Agent	Dose and Schedule	Duration of Therapy	Evidence Base	Comments
β-lactam tolerant: cefazolin	2 g IV every 6–8 h	Postinjury and then for 24 h following initial surgical management	Strong recommendation, low-quality evidence	Preferred regimen, recommendation based on contaminated H&N oncology and open fracture data, however
β-lactam allergic: clindamycin	600 mg IV every 8 h	Postinjury and then for 24 h following initial surgical management	Strong recommendation, low-quality evidence	Acceptable alternative to cefazolin

IV, intravenously; H&N, ●●●.

be warranted; however, the evidence remains very poor and further studies are needed.

Eye Injuries

Despite the historical risk of endophthalmitis with intraocular foreign bodies (IOFBs), Role 5 (i.e., fixed hospital in home nation) data from Walter Reed Army Medical Center reveal only one definite case of endophthalmitis since 2001 with more than 100 eyes sustaining IOFB injuries (Dr. Marcus Colyer, personal communication). Infections in tissues surrounding the eye demonstrate a similarly low rate of infection (Dr. Marcus Colyer, personal communication). No cases of bacterial corneal infections have been reported following trauma, while three eyes have suffered fungal keratitis following penetrating eye injuries (less than 1% incidence). Preseptal, orbital, and adnexal infection rates have similarly remained low.

METHODS

A literature search was conducted using health technology assessment resources, including, PubMed, Embase, and DTIC. The search was limited to English-language articles that were published between January 1, 2006, and November 30, 2010. Five independent reviewers screened articles using predefined criteria.

POSTINJURY ANTIMICROBIALS

Maxillofacial and Neck Injuries

Antibiotic prophylaxis for war injuries has been described using "cephalosporins" and continuing them for at least 3 days postoperatively with some success.[21] Perioperative ampicillin or penicillin[17] have also been used. We previously concluded these agents might have utility. However, the duration of therapy, the definition of infection, and the organisms encountered are not defined, and the evidence to support antibiotic prophylaxis use is poor. Perioperative antibiotics are clearly still needed for traumatic war wounds of the maxillofacial region as they present contaminated with oral secretions and environmental debris. A recent extensive review of antibiotics for facial trauma recommended limiting prophylaxis to patients who have gross wound contamination, open fractures, joint involvement, or require delayed wound closure; to patients who are immunocompromised or at high risk for endocarditis; and to patients having gunshot wounds or penetrating injuries from military weaponry.[23] Studies show reductions in contaminated surgery infection rates from 28% to 87% down to 6% to 20% using perioperative antibiotics, but these studies did not include trauma populations.[1] Suggested prophylactic agents are included in Table 1. T1,AQ:2

The optimal duration of perioperative coverage for contaminated combat trauma wounds remains undefined in the literature based on recent publications. In our last review, we concluded that data from contaminated major head and neck cancer surgery might be applicable to traumatic injuries because the majority of infections are polymicrobial in both settings, as are other factors such as impaired vascular flow, large tissue defects, etc.[1,24] A prospective randomized placebo-controlled multicenter trial of 1 day versus 5 days of antibiotics in this population showed 19% of patients infected with 1 day of coverage versus 25% with 5 days (not significant).[24] This study provides robust evidence that extending perioperative prophylaxis past 24 hours does not reduce infection rates and is probably unnecessary in maxillofacial and neck trauma surgery. Maxillofacial fractures result from trauma, and while not equivalent to combat injuries, also often become infected and require fixative surgery. Therefore, studies in this area might also help define optimal use of prophylactic antibiotics for war injuries. A recent systematic review of prophylactic antibiotics for facial fractures analyzed four studies of good quality.[25] The authors found that short-term prophylactic antibiotics in one study resulted in a fourfold reduction in infections[26] and calculated a threefold decrease in the infection rate when all four studies are combined.[25] This evidence supports continued use of perioperative antibiotics when conducting repair of maxillofacial fractures and suggests surgical debridement alone is inadequate. Furthermore, the systematic review concluded use of 1 day or one dose of antimicrobials was as effective as longer courses. Zygoma, maxilla, and condyle injuries did not become infected, whereas the mandible injuries did (29%), and the authors' final guidance was for short-term antibiotics for compound mandibular fractures and none for zygoma, maxilla, and condylar injuries. Caution is required in extrapolating these results to combat injuries; however, in these data, which represent only 1 study, the numbers are small and no high-velocity gunshots were analyzed.

A recent retrospective review of prophylactic antibiotics for zygomatic fractures from three civilian centers might be applicable to combat wounds; however, no gunshots were included.[27] The authors studied 134 patients and used a

protocol of no antibiotics if reduction was performed without plating; oral amoxicillin/clavulanate or cefuroxime or an ampicillin/dicloxacillin combination preoperatively and for two doses postoperatively if extra-oral reduction with plating was required; and the same regimen plus metronidazole if intraoral reduction with plating was required. This approach resulted in a 2% infection rate (higher than the previously quoted 0%), all in the intraoral fixation group. Notably, both infected patients did not receive metronidazole despite it being on their protocol. These studies suggest that shorter courses of antibiotics might be useful in combat injuries of the zygoma requiring fixation and that anaerobic coverage is important when the oral mucosa is involved. These conclusions are derived from experience with noncombat wounds and further studies are needed.

In summary, based on these recent[25,27] and previously reviewed studies[26,28,29] from mandibular fractures and contaminated head and neck cases[24] with similar outcomes, antibiotics in excess of those administered during the 24-hour perioperative period for maxillofacial injury do not appear to reduce wound infection (Table 1) and should be discontinued at 24 hours postoperatively.

Eye Injuries

Since 2001, endophthalmitis rates remain unusually low following combat ocular trauma. This has been attributed to the immediacy of globe repair and the universal administration of broad-spectrum prophylactic antibiotics. A prospective randomized study from Iran showed a statistically significant reduction in posttraumatic endophthalmitis rates when intraocular antibiotics were administered at the time of injury (2.3% vs. 0.3%).[30] Given the historical concern regarding the injection of intraocular antimicrobials (particularly gentamicin) in uninfected eyes, this route of administration is not currently recommended as the standard of care.[31] Instead, in select cases of extreme intraocular contamination, their use is at the treating ophthalmologist's discretion. Current treatment patterns dictate the initiation of a fourth-generation oral and topical fluoroquinolone (Table 2) for the prevention of ocular infection. Data from the 1980s suggested that systemic antimicrobials have no role in the prevention or treatment of endophthalmitis.[32] However, newer antimicrobials may provide improved intraocular penetration and are currently recommended in oral or intravenous routes in all cases of penetrating ocular trauma.[33,34] Suggested postinjury antimicrobial agents are included in Table 2.

DEBRIDEMENT AND IRRIGATION

Maxillofacial and Neck Injuries

There are no new studies in this area; however, acute management of most routine maxillofacial injuries should include wound debridement, primary closure, anatomic reduction, stabilization, and fixation of fractures. This is believed to result in an acceptably low rate of infection and return of form and function.[35,36]

Eye Injuries

Irrigation and debridement of the eye in the field (i.e., Role 1 "buddy care") or at Role 2 (e.g., Forward Resuscitative Surgical System) facilities are discouraged. The eye should be protected with a rigid eye ("Fox") shield by field medical teams, and early primary closure of wounds (within 6–8 hours) with careful wound debridement and placement of perioperative prophylactic subconjunctival antibiotics by an ophthalmologist at Role 3 is preferred.

Although more study is necessary to examine whether topical agents are effective, no further studies have presented themselves since our last review, therefore we maintain our conclusion that early globe closure with cleansing of wounds using irrigation and conservative debridement of devitalized tissue reduces foreign bodies and the bacterial load that contributes to postoperative infection. The recommended irrigation solution is balanced salt solution, but the most effective irrigation solution remains unclear.

SURGICAL WOUND MANAGEMENT

Maxillofacial and Neck Injuries

In our previous review,[1] a low infection rate for maxillofacial and neck injuries overall was attributed to aggressive debridement, irrigation of wounds, meticulous removal of contaminates, minimal introduction of foreign synthetic material during initial surgery, coverage of bone with tension-free closure when possible, and immediate institution of antibiotics in high-risk wounds. Management paradigms for maxillofacial and neck injuries have evolved over the last 50 years, and while the basic principles of wound management

TABLE 2. Suggested Antimicrobials and Duration of Administration for Postinjury Use in Eye Combat-Related Injuries

Agent	Dose and Schedule	Duration of Therapy	Evidence Base	Comments
Penetrating injury: β-lactam tolerant or allergic—levofloxacin	500 mg PO or IV daily	Postinjury and then for 7 d postoperatively or until retinal evaluation	Strong recommendation, low-quality evidence	Preferred regimen, recommendation based on retrospective trauma data and nontrauma studies of ocular penetration
Eye injury: burn or abrasion—erythromycin or bacitracin ophthalmic ointment, or fluoroquinolone ophthalmic solution	Topical: QID and PRN for symptomatic relief 1 drop QID	Until epithelium healed (no fluoroescein staining)	Strong recommendation, low-quality evidence	

PO, orally; IV, intravenously; QID, 4 times daily; PRN, as needed.

as outlined above generally apply to all sites in the head and neck, there remain some important differences based on the location of the skeletal injury and status of the soft tissue envelope.[35–37]

Facial Injuries

There is little controversy over the acute management of most routine maxillofacial injuries: postinjury antimicrobials, wound debridement, primary closure, anatomic reduction, stabilization, and fixation of fractures will result in an acceptably low rate of infection and return of form and function.[35–37] It is also accepted that for more significant high-energy trauma, early and conservative debridement, irrigation, fixation and immobilization, and primary closure with drainage are important to prevent infection.[38] However, there is no consensus regarding the optimal management of high-velocity injuries that result in severely comminuted mandibular fractures, either with or without composite tissue loss.

Some authors advocate closed reduction and delayed reconstruction as the preferred approach to the management of highly comminuted and avulsive mandibular fractures to prevent infection.[39] It appears that the loss of mucosal lining and difficulty in achieving a watertight intraoral soft tissue closure are associated with a high failure rate of primary mandibular bone grafts. Thus, grossly contaminated, avulsive defects of the mandible have been managed by stabilization of existing bone fragments, primary soft tissue closure, serial debridements, and a delay of bone reconstruction for at least 8 weeks.

The problem with this approach is that by delaying the restoration of ideal skeletal contours, projection, and symmetry, scar contracture occurs, and secondary reconstruction is compromised by an inelastic and hypovascular wound bed. To overcome these problems, some authors have advocated "temporary" wound coverage techniques and deferral of lengthy definitive procedures to a time when the patient has stabilized.[40] Other authors have proposed that severe facial trauma requires early tissue debridement and composite free tissue transfer to minimize scar contracture.[41] Indeed, a small study of immediate fixation versus delayed showed a 7% versus 43% infection rate.[19] Early reconstruction using microvascular free flaps facilitates early mucosal wound closure and could thereby decrease risk of delayed infection or fistula formation, but requires a commitment of significant resources and skills that may not be readily available at Role 3 facilities.

Regardless of the reconstruction method used, maxillofacial and neck wound beds will often require a period of intensive wound care before definitive restoration of form and function. Byrnside et al.[42] reported their use of negative pressure wound therapy (NPWT) for wounds of the head and neck to facilitate formation of soft tissue granulation and promote closure of challenging soft tissue defects. Their study does not present statistically significant conclusions, but is notable for introducing the "wound vac" (NPWT) for use in head and neck wounds and recognizing its potential to decrease the incidence of wound infections and affect outcomes. NPWT might have a role in prevention of infections of the maxillofacial and neck region, but the complex topography might make its application to the face difficult.

Neck Injuries

Recent changes in the evaluation and surgical management of combat wounds of the neck may affect subsequent infection rates. Imaging technology advancements, particularly computed tomographic angiography (CTA), are altering the management of patients with penetrating neck injuries. Helical and multislice CTA has emerged as a fast, minimally invasive study to evaluate penetrating neck injuries.[43–45] CTA is readily available in most trauma centers and Role 3 deployed hospitals, it allows accurate evaluation of the vascular and extravascular soft tissues and bones in less than 3 minutes, and it does not require the support of additional nonphysician staff. Direct and indirect signs of vascular injury are well demonstrated, as are signs of violation of the aerodigestive tract, neurologic injury, and bony fracture.

Although some centers still practice routine exploration for all neck injuries penetrating the platysma, many civilian centers in the United States have adopted a policy of selective exploration based on clinical and radiographic examination.[46,47] In a retrospective study of 65 patients (47% gunshot wounds) seen at a civilian trauma center between 2000 and 2005 with neck wounds that penetrated the platysma, Bell et al.[48,49] found that increased use of CTA in hemodynamically stable patients was associated with a decreased frequency of neck exploration and a "virtual elimination of negative neck exploration" The surgical approaches described were standard. However, data on antimicrobial or surgical drain use, length of follow-up, or detailed patient outcomes were not provided. No comparison between operated and observed patient outcomes was included, but the authors concluded that selective surgical intervention for these injuries resulted in minimal morbidity (including a low 3% infection rate) and mortality at their institution. While no patients with combat wounds were included in this trial, almost half were gunshot victims, and we concur with recommendations that CTA be considered in early management of combat wounds to the neck. Further study is indicated to determine the effect of reducing exploratory surgery on infectious complications.

When upper aerodigestive tract injury is suspected, diagnostic workup should be expeditious as management delayed by more than 24 hours increases morbidity and mortality.[50] Delay in diagnosis of esophageal perforation is a particularly important predictor of infectious complications. When an esophageal injury is found early, surgical management should include copious wound irrigation, cautious debridement, a two-layer closure, and adequate drainage. After repair of the mucosal perforation, a muscle flap should be placed over the esophageal suture line for further protection. If an extensive esophageal injury is present, a lateral cervical esophagostomy should be created and definitive repair performed later.

If suspicion of a pharyngeal perforation remains despite being unconfirmed by examination or exploration, the casualty should have nothing by mouth, be observed for 7 days, and a swallow study should be repeated before advancing the diet. Fever, tachycardia, or widening of the mediastinum on

serial chest radiographs or computed tomography indicates the need for repeat endoscopy or neck exploration.

Eye Injuries

Ocular injuries remain unique with regard to prevention and treatment of infection insofar as the majority of the eye is avascular and has limited capability to counter the presence of even a small bacterial load. Risk factors for the development of endophthalmitis include delayed primary closure, presence of IOFB, violation of the lens capsule, and wound contamination.[51] Thus, treatment paradigms have evolved during the current conflict to emphasize immediate protection of the eye with a Fox Shield by field medical teams, early primary closure of wounds (within 6–8 hours) with careful wound debridement and placement of perioperative prophylactic subconjunctival antimicrobials at Role 3.[34] Given the low infection rate, the need to urgently evacuate patients to Role 4 (e.g., fixed hospital out of combat theater of operations) and Role 5 has superseded the urgency of IOFB removal with the known surgical complexities of vitreoretinal intervention in an austere environment.

Aggressive debridement of lid wounds with reapproximation of margins and placement of nasolacrimal stents have been the mainstay in the surgical management of periocular wounds and likely accounts for low rate of extraocular infections and should be the standard of care.[52,53]

UNRESOLVED ISSUES/RESEARCH GAPS

Since publication of the last guidelines in 2008, no new epidemiologic studies of bacterial etiologies or antimicrobials used have been published for infections following eye, maxillofacial, or neck trauma. These studies, if performed, would be helpful in formulating better guidance for empiric antimicrobial coverage following injury and assessing best practices of antimicrobial use. What can be said is that ocular infections remain extremely rare and that current practice of eye injury management throughout all roles of care should continue. The limited reports of pathogens isolated in the Vietnam, Lebanese, and Balkans conflicts indicate that these data are collectable. We encourage military and civilian clinicians who manage gunshot wounds and blast injuries to undertake, at a minimum, retrospective studies of bacterial epidemiology and antimicrobial usage in comparison with outcomes using existing databases and records.

Recent changes in surgical technique include a debate over whether outcomes are improved in delayed versus immediate reconstruction. Based on one study, CTA appears to reduce unnecessary neck exploration and subsequent infection and therefore should be strongly considered as part of initial management of penetrating neck trauma.

No new randomized controlled trials for postinjury antimicrobial prophylaxis of craniomaxillofacial trauma have been published since our last review. Several publications in the facial fracture literature (which includes trauma patients who often develop infection), while not equivalent to combat injury, seem to reinforce what has been learned in contaminated head and neck surgery. Longer periods of postoperative antimicrobials do not appear better than shorter regimens in preventing postoperative infections. Therefore, we continue to recommend stopping postinjury antimicrobial therapy 24 hours after initial surgical management.

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Murine Immune Response to a Chronic *Staphylococcus aureus* Biofilm Infection▽†

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Staphylococcus aureus **has reemerged as an important human pathogen in recent decades. Although many infections caused by this microbial species persist through a biofilm mode of growth, little is known about how the host's adaptive immune system responds to these biofilm infections. In this study, *S. aureus* cells adhered to pins in culture and were subsequently inserted into the tibiae of C57BL/6 mice, with an infecting dose of 2×10^5 CFU. This model was utilized to determine local cytokine levels, antibody (Ab) function, and T cell populations at multiple time points throughout infection. Like human hosts, *S. aureus* implant infection was chronic and remained localized in 100% of C57BL/6 mice at a consistent level of approximately 10^7 CFU/gram bone tissue after day 7. This infection persisted locally for >49 days and was recalcitrant to clearance by the host immune response and antimicrobial therapy. Local inflammatory cytokines of the Th1 (interleukin-2 [IL-2], IL-12 p70, tumor necrosis factor alpha [TNF-α], and IL-1β) and Th17 (IL-6 and IL-17) responses were upregulated throughout the infection, except IL-12 p70, which dwindled late in the infection. In addition, Th1 Ab subtypes against a biofilm antigen (SA0486) were upregulated early in the infection, while Th2 Abs and anti-inflammatory regulatory T cells (Tregs) were not upregulated until later. These results indicate that early Th1 and Th17 inflammatory responses and downregulated Th2 and Treg responses occur during the development of a chronic biofilm implant infection. This unrestrained inflammatory response may cause tissue damage, thereby enabling *S. aureus* to attach and thrive in a biofilm mode of growth.**

One of the most common and costly problems for the U.S. health care system is nosocomial infections (22), with *Staphylococcus aureus* being the second leading cause of such infections (5). Methicillin-resistant *S. aureus* (MRSA) is responsible for 40 to 60% of all nosocomially acquired *S. aureus* infections, and these resistant strains are now considered to be endemic in the hospital setting (29). Community-associated *S. aureus* strains may also acquire methicillin resistance (community-associated MRSA [CA-MRSA]), and the modern emergence of such strains is of great concern (18, 23, 47).

Recent studies indicate that *S. aureus* is also the major mediator of prosthetic implant infection (1, 38). The increasing involvement of *S. aureus* in foreign body-related infections, the rapid development of resistance to multiple antibiotics by these organisms, and the propensity of these infections to change from an acute infection to one that is persistent, chronic, and recurrent have led to this organism once again receiving significant attention.

Treatment of prosthetic implant infections is a complicated process, and a number of staphylococcal defense mechanisms may be responsible for this difficulty, as well as the capacity of *S. aureus* to evade clearance by the host immune response. One of the most important mechanisms utilized by *S. aureus* to thwart the host immune response and develop into a persistent infection is the formation of a highly developed biofilm. A biofilm is defined as a microbe-derived community in which bacterial cells are attached to a hydrated surface and embedded in a polysaccharide matrix (13). Bacteria in a biofilm exhibit an altered phenotype in their growth, gene expression, and protein production (15), and prosthetic medical devices are often a site of chronic infection because they present a suitable substrate for bacterial adherence, colonization, and biofilm formation. Biofilm formation by *S. aureus* during prosthetic implant infection makes eradication of this bacteria extremely difficult, due in part to the dramatically increased resistance of bacteria in a biofilm to host defenses (16) and to antibiotics (35, 36) compared to that of their planktonic counterparts.

S. aureus elicits a strong inflammatory response, resulting in the migration of large numbers of neutrophils and macrophages to the site of infection. A majority of *S. aureus* strains have been shown to elicit the production of interleukin-1β (IL-1β), IL-6, and IL-12 p70 in monocytes *in vitro*, and this may result in biasing the immune response toward a Th1-type response *in vivo* (33). Staphylococcal enterotoxin B (SEB) has been shown to induce *in vitro* expression of IL-2 and gamma interferon (IFN-γ) with rapid and intermediate kinetics, respectively, but slow expression of IL-10, a Th2 cytokine (2). The superantigen, staphylococcal enterotoxin A (SEA), also elicits a strong Th1 response *in vitro*, with concomitant production of tumor necrosis factor alpha (TNF-α) and MIP-1α (14). Another staphylococcal toxin, alpha-toxin, has been

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shown to increase IFN-γ production in $CD4^+$ T cells *in vitro* and to increase binding of DNA to T-bet, a transcription factor involved in commitment to a Th1 response (7). Protein A is also a potent inducer of Th1 cytokines such as IFN-γ, TNF-α, and IL-1 in mice receiving intraperitoneal (i.p.) injections of protein A (42).

While the studies described above hint that a Th1-biased adaptive immune response could result from *S. aureus* infection, relatively little is known about Th2, Th17, and regulatory T cell (Treg) responses in an *in vivo* model of *S. aureus* biofilm infection. Also, it is unknown how the host immune system responds to *S. aureus* as it progresses from an acute to a chronic infection that resists clearance by the host immune system. Determination of the phenotypic characteristics and activation states of infiltrating immune cells, IgG subisotypes produced, and cytokines elicited during acute and chronic *S. aureus* infection may provide insight into the mechanisms of immune evasion used by *S. aureus* to establish a chronic biofilm infection. This knowledge will also further our understanding of why the host does not mount an effective immune response and is ineffective in the clearance of this pathogen.

An augmented Th2 response was previously shown to be effective at preventing a biofilm infection in the early phase of formation (34, 41, 44). However, this antibody (Ab)-mediated response may be downregulated both by the early host cytokine response to *S. aureus* infection and by the *S. aureus* superantigens, capsule, and other toxins, but this has not been previously studied *in vivo*. Although a recent mouse model of prosthetic implant infection was developed (28), the resulting infection did not exhibit all of the hallmarks of a true biofilm infection, as the host and antibiotic therapy were able to clear the infection. Therefore, we developed a mouse model of biofilm infection that is recalcitrant to the host immune response and antimicrobial agent clearance.

In order to characterize the host cellular, Ab, and cytokine responses to *Staphylococcus aureus* biofilm-mediated implant infections, we adapted a mouse model of *S. aureus* implant infection using a biofilm-forming strain of MRSA isolated from an infected prosthetic implant. Biofilms were formed on stainless steel pins and then implanted into C57BL/6 mice (12). After implantation with pins with adherent *S. aureus* cells, viable bacteria could be cultured from the infected pin at 49 days postinfection, even in the presence of vancomycin. In addition, imaging studies demonstrated a well-developed biofilm on the infected pins, thus indicating the development of a chronic biofilm-mediated implant infection. In C57BL/6 mice, implantation of *S. aureus*-coated pins led to the activation of a CD4 response and the early production of IgG2b (the dominant Th1-associated IgG subtype) against the biofilm-upregulated antigen SA0486 (6). In addition, Th1 and Th17 cytokines were present at the implant site, and Tregs were suppressed early in the infection. These studies suggest that staphylococcal infection resulted in the skewing of the host immune response toward proinflammatory Th1 and Th17 responses, which fail to clear the infection.

S. aureus biofilm infections present a very serious and costly problem. Furthering our knowledge of how the host immune system fails to clear this pathogen will help the scientific community to find better control and therapeutic strategies.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 mice (6 to 8 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained under microisolator conditions in the animal facility at the University of Maryland School of Medicine (Baltimore, MD), in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

Bacterial strain and preparation of implants. The strain of *S. aureus* used in these experiments, MRSA-M2 (M2), is a clinical isolate obtained from an osteomyelitis patient undergoing treatment at the University of Texas Medical Branch (Galveston, TX) and has been used in previous biofilm molecular analyses and animal infection models (6, 26, 32, 39). The well-characterized *S. aureus* strain UAMS-1 was also used in infection studies and antibody isotype studies (see below) in order to ensure that the results were not limited to the M2 strain (4, 10, 11, 17, 37, 43). Autoclaved 0.25-mm insect pins (Fine Science Tools, Foster City, CA) were incubated for 2 h in 10 ml of an overnight culture of *S. aureus* that was diluted 1:100 in sterile Trypticase soy broth.

Cloning, expression, and purification of proteins. Candidate antigens selected by Brady et al. (6) were amplified using the following primers: 5′-ACTCTAGG TCTCACTCCAAAGAAGATTCAAAAGAAGAACAAAT-3′ and 5′-ATGGT AGGTCTCATATCAGCTATCTTCATCAGACGGCCCA-3′. The PCR products were cloned into pASK-IBA14, transformed into *Escherichia coli* TOP10, and sequenced. The clones were then expressed using anhydrotetracycline induction. SA0486 was purified via Strep-Tactin Superflow columns (IBA, Göttingen, Germany). Purity was confirmed by resolving each protein on 15% SDS-PAGE, and quantities were determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL).

Surgical implantation of pins. Four to eight mice per experimental group received tibial implants. Mice were anesthetized via i.p. injection of 100 mg ketamine/kg of body weight (Ketaset; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and 10 mg xylazine/kg (Rugby Laboratories, Inc., Rockville Center, NY). The left leg of each mouse was cleansed with povidone iodine and rinsed with 70% ethanol before surgical implantation of an *S. aureus*-coated or uninfected control pin, according to the methods previously described by Li et al. (28). For antimicrobial efficacy experiments, mice were treated via subcutaneous (s.c.) injection of 50 mg of vancomycin/kg twice daily for 10 days beginning on day 14 postimplantation, which is approximately 10-fold higher than that used in previous studies (9, 24, 45). All other mice did not undergo any additional treatments after surgery until sacrifice. All animal experiments were performed in accordance to protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland School of Medicine (Baltimore, MD). Nonimplanted 0.5-mm sections of pins incubated with *S. aureus* were homogenized and cultured to determine the infecting dose upon pin implantation. It was determined that approximately 2×10^5 CFU/pin section (standard deviation [SD] = 5×10^4) was delivered to the tibia for infection.

Bone cultures. At 4, 7, 14, 21, 28, and 49 days postimplantation, infected and uninfected mice were euthanized, left tibiae were removed, and all soft tissue was dissected from the bone. Using sterile scissors, tibiae were cut into small pieces and placed in 300 μl of 0.85% sterile saline per 100 μg of bone. Bones were homogenized using a Polytron PT 1200 handheld homogenizer (Kinematica, Bohemia, NY), and serial 10-fold dilutions of bone homogenates were plated on sheep's blood agar plates to enumerate the number of viable *S. aureus* cells per g bone. Additionally, 0.5-mm sections of pins representing the lengths inserted into the tibiae of mice were incubated with *S. aureus* as described above and processed for culture in order to determine the infecting dose.

PNA-FISH biofilm detection on explanted pins. Infected and uninfected sterile pins were inserted into the tibia of mice. Pins were carefully removed from the tibiae of infected and uninfected mice to prevent perturbation of biofilm mass at 7 and 21 days postimplantation. Pins were placed in Eppendorf tubes and fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) before peptide nucleic acid fluorescent *in situ* hybridization (PNA-FISH) with a fluorescein isothiocyanate (FITC)-labeled *S. aureus* probe and a rhodamine-labeled universal eukaryotic cell probe, as per the manufacturer's instructions (AdvanDx, Woburn, MA). Each pin was then examined with a Zeiss LSM 510 confocal scanning laser microscope (Carl Zeiss, Thornwood, NY) for both green and red fluorescence using a FITC/Texas Red dual-band filter and a 63× objective.

Measurement of serum IgG subisotype level. Blood samples obtained from mice that had received tibial implants were collected at 0, 7, 14, 21, and 28 days postimplantation, allowed to clot at room temperature for 20 min, and then centrifuged at $4{,}000 \times g$ for 15 min. Sera were separated from clotted cells and stored at −70°C until ready for use. A high-binding-capacity 96-well enzyme-linked immunosorbent assay (ELISA) plate (Becton Dickinson, Bedford, MA)

was coated with 100 μl of antigen/well (100 ng/well) diluted in PBS and incubated overnight at 4°C. After the wells were washed three times with PBS containing 0.5% Tween 20 (PBST), nonspecific binding activity was blocked by addition of 200 μl of 1% BSA/well and incubation at room temperature for 2 h. After wells were washed three times with PBST, test sera were added at the appropriate dilutions and incubated for 1 h at room temperature. After sample incubation, wells were washed three times with PBST. Rabbit anti-mouse IgG1, IgG2a, and IgG2b secondary Abs (Invitrogen, Carlsbad, CA) were added to the appropriate wells and incubated at room temperature for 1 h. Following five washes with PBST, plate-bound IgG1, IgG2a, and IgG2b were detected with 50 μl of affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA)/well. The colorimetric reaction was developed using OptEIA (BD Biosciences, San Jose, CA) as the substrate (50 μl/well), and color intensity was read at 450 nm. Color intensity was compared to a standard curve for each IgG subtype, and results were expressed as the number of pg/ml.

Measurement of cytokine levels at the implant site. Implanted tibiae and the surrounding soft tissue were harvested from mice at days 7 and 28 postimplantation and stored at −70°C. Samples were homogenized on ice in sterile PBS containing an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Tissue homogenates were centrifuged for 15 min at 14,000 × *g* at 4°C, and supernatants were analyzed by the Cytokine Core Laboratory at the University of Maryland School of Medicine (Baltimore, MD) using quantitative multiplex sandwich ELISA technology. Cytokines tested included murine IL-2, IL-4, IL-6, IL-10, IL-12 p70, IL-17, and TNF-α.

$CD4^+$ and $CD8^+$ frequency analysis by flow cytometry. Draining lymph node (LN) cells from mice were harvested at days 4, 7, 14, 21, and 28 postimplantation, and single-cell suspensions were prepared. To determine the $CD4^+$ and $CD8^+$ T cell frequency, 1×10^6 LN cells were aliquoted into fluorescence-activated cell sorter (FACS) tubes (Becton Dickinson, Bedford, MA) and surface stained with FITC-labeled anti-mouse CD3, phycoerythrin (PE)-labeled anti-mouse CD8, peridinin chlorophyll protein (PerCP)-labeled anti-mouse CD4, and allophycocyanin (APC)-labeled anti-mouse CD44 MAbs (BD Biosciences, San Diego, CA). Cells were analyzed using either a FACScan or an LSR II flow cytometer (BD Biosciences, San Jose, CA), and results were expressed as the percentages of $CD4^+$, $CD8^+$, and $CD44^+$ cells after gating on the lymphocyte population.

Treg frequency analysis by flow cytometry. For Treg analysis, 1×10^6 draining LN cells were aliquoted into FACS tubes (Becton Dickinson, Bedford, MA), and a Treg FACS staining kit (eBioscience, San Diego, CA) was used to determine the frequency of Tregs in draining LN cells. Cells were surface stained with FITC-labeled anti-mouse CD4 and PE-labeled anti-mouse CD25 MAbs and stained intracellularly with PE-Cy5-labeled anti-mouse Foxp3 MAb, in accordance with the manufacturer's protocol. Results were expressed as ratios of the frequency of $Foxp3^+$ $CD25^+$ $CD4^+$ T cells in infected mice to that in uninfected mice. Cells were analyzed using either a FACScan or an LSR II flow cytometer (BD Biosciences, San Jose, CA).

Statistical analysis. Mean and SD values were calculated and analyzed using Student's *t* test, with a *P* value of <0.05 to determine statistical significance. Experiments determining the percentages of mice still infected after vancomycin or PBS treatment were analyzed using Fisher's exact test, with a *P* value of <0.05 to determine statistical significance.

RESULTS

***S. aureus* implant infection results in chronic infection.** Tibiae from mice receiving implants of *S. aureus*-coated and control sterile pins were harvested and processed at days 4, 7, 14, 21, 28, and 49 postimplantation. The numbers of CFU were enumerated from homogenized bone tissue to determine the development of chronic infection and bacterial loads in the tibiae. Results demonstrate that viable *S. aureus* cells were cultured from the infected pin and surrounding bone at all time points tested, as far out as 49 days postinfection (Fig. 1). Bacterial loads initially increased to over 3 logs of the infecting dose to $>10^8$ CFU/tibia but then decreased between 4 and 7 days postinfection. However, at day 7 and beyond, bacterial loads were consistent. Biofilm formation was evident on implanted pins from infected (Fig. 1B) but not uninfected mice (Fig. 1C and D) by confocal scanning laser microscopy. In addition, vancomycin treatment did not clear infection in any of the mice receiving *S. aureus*-coated implants, even though the M2 strain of *S. aureus* is susceptible to vancomycin in planktonic culture. In order to demonstrate that this infection modality was not unique to the M2 clinical strain of *S. aureus*, we also infected mice with UAMS-1, a well-characterized strain derived from osteomyelitis patients (4, 10, 11, 17, 37, 43). This strain was able to produce an implant infection with the similar trend and bacterial concentrations of the chronic infection seen with *S. aureus* strain M2 (see Fig. S1 in the supplemental material).

***S. aureus* implant infection elicits a local CD4 T cell response but not a CD8 T cell response.** Draining LN cells were harvested from mice at 4, 7, 21, and 28 days postimplantation. A single-cell suspension was prepared, and cells were FACS stained to determine $CD4^+$ and $CD8^+$ T cell frequencies and the upregulation of CD44, a classic marker of T cell activation. Results demonstrate that in the draining LN cells of infected mice, there is a significantly higher frequency of $CD4^+$ cells than that in draining LN cells of uninfected mice. This difference was not observed when comparing $CD8^+$ frequencies in draining LN cells of infected mice versus those of uninfected mice (Fig. 2A and B). Further, in infected mice, there is a significant upregulation of CD44 only in the $CD4^+$ population of LN cells. This upregulation of CD44, indicating T cell activation, is not observed in $CD8^+$ LN cells from infected mice (Fig. 2C). In addition, there is no difference in the proportions of $CD44^+$ LN cells observed in either T cell population in uninfected mice (data not shown). At day 7 postinfection, there is a dramatic decrease in both CD4 and CD8 T cells in the draining lymph nodes of infected mice. This is potentially due to activation-induced cell death following polyclonal activation of T cells by *S. aureus* superantigens such as toxic shock syndrome toxin (TSST), which is produced by the MRSA-M2 strain used in this study.

***S. aureus* implant infection elicits differential production of Th1- and Th2-dependent subisotypes against a biofilm-upregulated antigen.** Levels of various IgG Ab subisotypes against the biofilm-upregulated antigen SA0486 were assessed using the commercially available Mouse Typer isotyping kit. SA0486, a staphylococcal lipoprotein of unknown function which is upregulated in *S. aureus* biofilms, was previously determined by our lab to be both immunogenic and expressed by *S. aureus* during the biofilm mode of growth *in vivo* (24). Sera from infected and uninfected mice collected at days 0, 7, 14, 21, and 28 postinfection were tested in this assay. ELISA plates were coated overnight with 0.1 μg of recombinant SA0486/well, and a conventional ELISA was performed to determine the levels of serum IgG1, IgG2a, and IgG2b Abs produced against SA0486. Early during implant infection, at day 7, there were significantly higher levels of IgG2b (a Th1-dependent subisotype) in the sera compared to those of IgG1 (a Th2-dependent subisotype). By day 28, however, there was a major decline in IgG2b levels and a concomitant increase in the levels of IgG1 (Fig. 3). These data indicate that both Th1- and Th2-dependent IgG subisotypes are produced in response to SA0486 by mice receiving infected implants. However, the kinetics of Ab production differ, with the Th1 IgG subisotypes (IgG2b-recognizing microbial polysaccharides) being produced early in the infection and the Th2 IgG subisotype (IgG1-recognized microbial surface proteins) having a delayed pro-

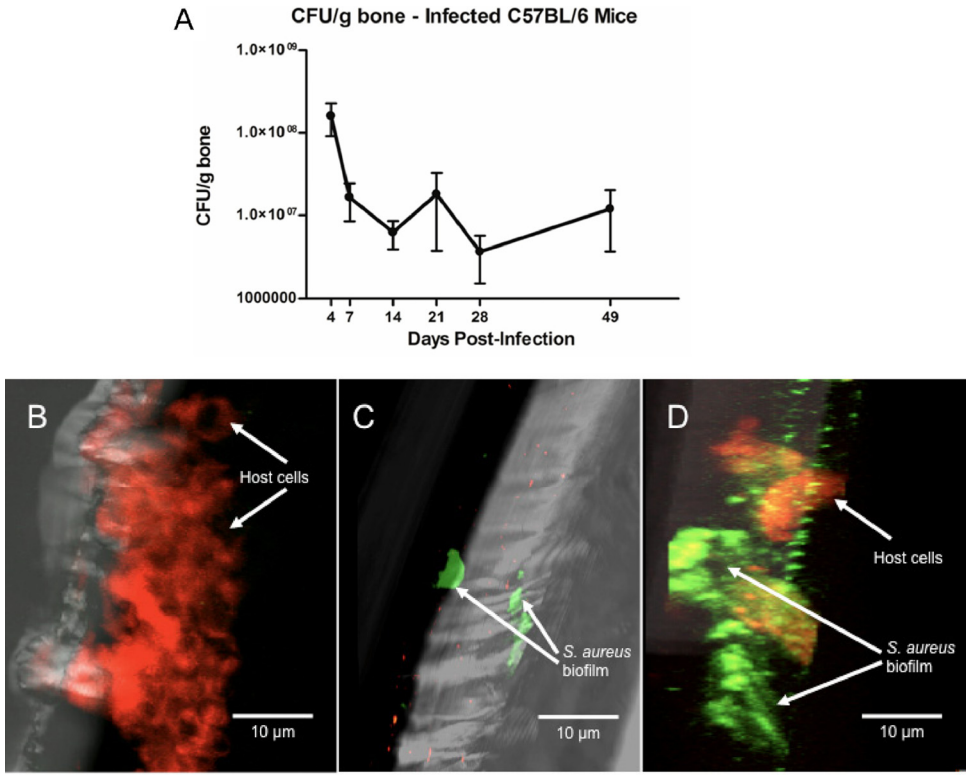

FIG. 1. (A) Development of chronic, biofilm-mediated infection that is recalcitrant to antimicrobial therapy. Number of CFU/g bone over time, indicating the development of a chronic infection. Tibiae from infected and uninfected mice were removed at 4, 7, 14, 21, 28, and 49 days postinfection. No CFU were found in uninfected mice. Serial dilutions of bone homogenates were plated on blood agar plates. The numbers of CFU/g bone were calculated and plotted over time. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Fisher's exact test. Bars represent SDs. (B to D) Confocal scanning laser microscopic images of uninfected pins removed at 21 days postimplantation (B) and *S. aureus*-infected pins removed at 7 (C) and 21 (D) days postimplantation. Pins were labeled using a FITC-labeled PNA-FISH probe. Biofilm formation is evident on the pin removed from the infected mouse.

duction, coming too late for clearance by the host. We also analyzed the IgG subtypes at multiple time points following infection using a well-characterized strain of *S. aureus*, UAMS-1 (see Fig. S2 in the supplemental material). Infection with the UAMS-1 strain also produces a similar IgG response, in which IgG2A and -B are first activated and it is not until day 21 that the IgG1 subtype begins to increase. While this response to UAMS-1 seems to be somewhat delayed compared to the response to the M2 strain, possibly due to strain-to-strain differences, the trend of an early Th1 Ab response followed by a delayed Th2 response is similar and confirms the response seen in the M2 clinical isolate.

***S. aureus* implant infection elicits Th1 cytokines at 14 days postinfection.** At days 7 and 28 postimplantation, tibiae were removed from infected and uninfected mice. Tibiae were homogenized, and cytokine levels in supernatants were analyzed. Results indicate the significant upregulation of several Th1 cytokines at day 7, including IL-2, IL-12 p70, and TNF-α. There is also significant upregulation of the Th17-associated cytokines IL-6 and IL-17 (Fig. 3). At day 28 postimplantation, the levels of several cytokines drop off, but there is still significantly greater production of IL-2, TNF-α, IL-6, and IL-17 (Fig. 4B).

***S. aureus* implant infection decreases the frequency of local Tregs.** To evaluate the levels of Tregs in the development of chronic implant infection, draining LN cells from infected and uninfected mice were harvested at 4, 7, 14, 21, and 28 days postinfection, and single-cell suspensions of LN cells were prepared. LN cells were FACS stained for CD4 and CD25 surface markers and for intracellular FoxP3. Results indicate that the percentage of $CD4^+$ T cells expressing Foxp3 was significantly lower at day 7 postimplantation in mice receiving *S. aureus*-coated implants rather than sterile implants (Fig. 5).

DISCUSSION

It is well established that growth in the biofilm state protects *S. aureus* from clearance by the host immune system. However, cellular and humoral responses to whole-cell *S. aureus* biofilm-mediated implant infections have not been characterized *in vivo*. Determination of the cell types, phenotypic characteristics, and activation states of infiltrating immune cells, as well as the cytokines and Ab subisotypes elicited during acute and chronic *S. aureus* infection, may provide insight into how *S. aureus* evades the host immune response in order to establish a chronic biofilm infection. This present study sought to adapt a mouse model of *S. aureus* biofilm-mediated prosthetic implant infection to accurately mimic chronic infection in humans and to characterize the cell-mediated immune response against this type of infection. We have demonstrated that our model

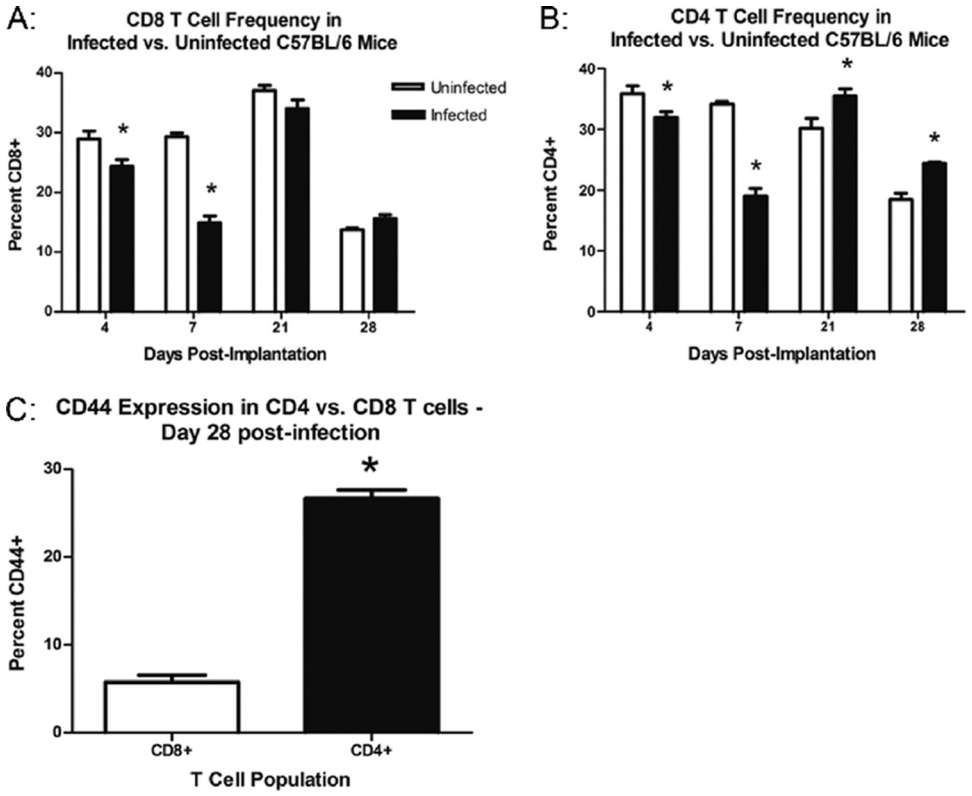

FIG. 2. T cell response to chronic implant infection. Draining lymph nodes were removed from infected and uninfected mice at 4, 7, 21, and 28 days postimplantation. Single-cell suspensions were stained as described in Materials and Methods. (A, B) CD8 (A) and CD4 (B) frequencies were determined by FACS analysis. Both populations are significantly decreased at days 4 and 7, likely due to activation-induced cell death following superantigen activation. At later time points, only the frequency of CD4 T cells is significantly increased in infected versus uninfected mice. (C) At day 28 postimplantation, there is increased expression of the activation marker CD44 only on CD4 T cells from infected mice. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test or Fisher's exact test. Bars represent SDs.

results in a chronic, localized infection in C57BL/6 mice that is recalcitrant to treatment with antibiotics or clearance by the host, similar to *S. aureus* prosthetic implant infections in patients. This infection was shown to elicit mainly Th1 and Th17 responses, while Treg responses were suppressed in infected C57BL/6 mice early during infection.

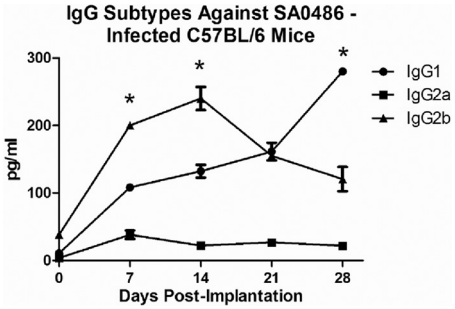

FIG. 3. IgG subtypes against the biofilm-upregulated antigen SA0486. Infected mice were bled at 0, 7, 14, 21, and 28 days postinfection. Sera were collected, and pooled serum samples were analyzed for levels of the Th2 antibody IgG1 and the Th1 antibodies IgG2a and IgG2b against SA0486 over time. IgG2b peaks early during infection on days 7 and 14, whereas IgG1 peaks much later on day 28, by which time mature biofilm formation and chronic infection have developed. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ for IgG1 compared to IgG2b by Student's t test. Bars represent SDs.

In the present study, the implantation of a pin coated with *S. aureus* grown under biofilm-forming conditions results in an infection that is recalcitrant to clearance by both the host immune response and vancomycin treatment. Following implant infection, the acute infection transitioned to a stable chronic infection by day 14 postinfection (Fig. 1). The decrease in CFU counts seen after the early acute expansion of *S. aureus* numbers was likely due to the native immune response killing planktonic bacteria in and around the implant site, since this was before an adaptive immune response was possible. However, during the chronic infection stage, *S. aureus* developed into a mature biofilm and CFU levels plateaued because the immune system is no longer able to clear these biofilm-bound bacteria. In addition, decreased clearance with the antimicrobial therapy of vancomycin confirms that the infection model is clinically relevant, since this is a hallmark of biofilm-mediated prosthetic implant infections in animal models of infection and human patients. Confocal scanning laser microscopy images of infected pins removed from tibiae at 7 and 21 days postimplantation provide further evidence of the presence of chronic infection and biofilm formation, as indicated by the presence of fluorescent green-labeled cocci and biofilm masses on infected (Fig. 1C and D) versus uninfected (Fig. 1B) pins.

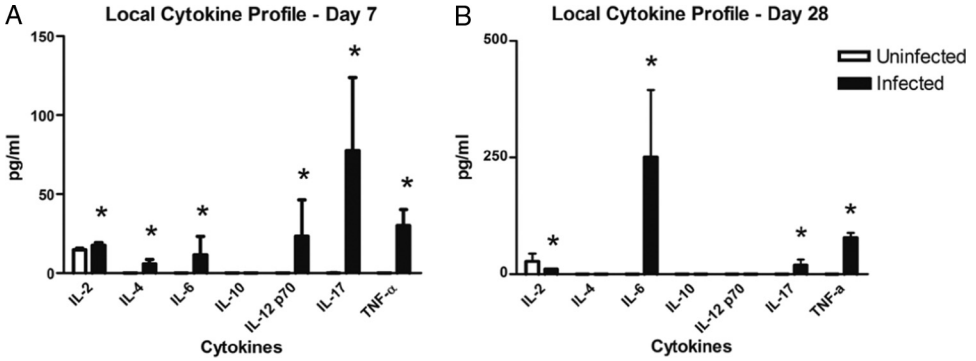

FIG. 4. Local cytokine profile at implant site. Tibiae were removed from mice receiving *S. aureus*-coated or sterile pins. Supernatants from bone homogenates were analyzed for cytokines at day 7 (A) and day 28 (B) postimplantation, as described in Materials and Methods. Significant upregulation of IL-2, IL-6, IL-12, IL-17, and TNF-α indicate a predominantly Th1- and Th17-type response. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test. Bars represents SDs.

Other data collected during this study, including T cell subtypes, support the hypothesis that the host responds with a CD4 T cell-mediated response (Fig. 2A and B). Additional support is provided by data derived from the Ab subtype experiments. During the early stages of prosthetic implant infection, the CD4 T cell response is mainly of a Th1 type, as indicated by the IgG subtypes produced against the *S. aureus* biofilm-upregulated antigen SA0486. The host humoral immune response to SA0486 results in IgG2b Abs (Fig. 3), the dominant IgG subtype associated with a Th1 response. These IgG2b Abs may not be able to effectively clear bacteria that are beginning to develop into a biofilm infection, allowing them to form a mature and chronic biofilm. This may be due in part to the poorly opsonizing characteristics of IgG2b Abs (27). Although the immune response does eventually mount a Th2-type response, indicated by the late production of IgG1, this switch does not occur until 28 days postinfection. By this point, IgG1 Abs, which may have successfully cleared an early biofilm, are incapable of clearing the mature biofilm that has formed on the implant and surrounding dead bone.

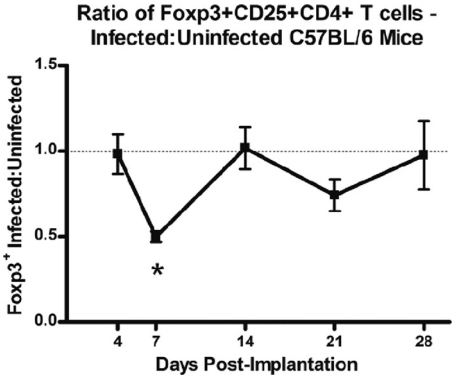

FIG. 5. Treg responses to *S. aureus* implant infection. Draining lymph nodes were removed from infected and uninfected mice at 4, 7, 14, 21, and 28 days postimplantation. Single-cell suspensions were intracellularly stained for Foxp3, as described in Materials and Methods. The Treg frequency, expressed as the ratio of Foxp3 expression in $CD4^+$ lymphocytes of infected mice to that of uninfected mice, was significantly reduced at day 7 postimplantation in mice receiving *S. aureus*-coated implants. $n = 5$ to 8 mice per group. Experiments were performed in triplicate. *, $P < 0.05$ compared to controls by Student's t test. Bars represent standard errors of the means.

The Th1 response early after implant infection that is associated with the development of a mature biofilm formation and damage to the host through the action of proinflammatory cytokines is further demonstrated by the cytokine response profile. At day 7 postimplantation, there was significant production of cytokines of the Th1 response (Fig. 4A) at the site of infection. While TNF-α levels continued to be elevated, the Th1 cytokine IL-12 p70 became undetectable (Fig. 4B). The later absence of IL-12 is likely due to the fact that this cytokine is usually expressed early during a Th1 response.

In these cytokine profile studies, there was also significant upregulation of the Th17-associated cytokines IL-6 and IL-17 at both early and late time points postimplantation, indicating that these mice are also mounting a robust Th17 response. Although Th17 cells play an integral role in clearing extracellular bacteria, several studies suggest that the Th17 response and resulting neutrophil activation are detrimental to the host during a biofilm-mediated infection (3, 25, 46). Biofilm-embedded bacteria are largely protected from neutrophil killing, and the concomitant release of inflammatory cytokines from these cells leads to the damage of host tissues and further devitalized surface biofilm formation. In addition, IL-6 has been implicated in promoting Treg insufficiency induced by staphylococcal enterotoxin B *in vitro* (48), thereby allowing the inflammatory response to go on unchecked, as discussed below. There was also a small upregulation of IL-4 early in the infection at day 7. Although IL-4 is a Th2 cytokine, this small and transient increase may not be physiologically relevant.

The last subset of the CD4 T cell-mediated response, Tregs, exhibits anti-inflammatory effects through the suppression of proinflammatory $CD4^+$ T effector cells. Therefore, we hypothesized that *S. aureus* may also target this lymphocyte population during the development of chronic implant infection. In the case of infection with *S. aureus*, Tregs are capable of modulating inflammation induced by staphylococcal enterotoxins, such as SEB (21), but their activity is also actively suppressed by this toxin during infection *in vitro* (8, 21, 48). Our data indicate that *S. aureus* implant infection leads to a significant decrease in the frequency of $Foxp3^+$ $CD4^+$ Tregs in the drain-

ing LN cells of infected versus uninfected mice at day 7 postimplantation (Fig. 5). This early downregulation of Tregs by *S. aureus* can further enhance the ability of *S. aureus* to produce proinflammatory Th1 and Th17 immune responses. Taken together, the activation of the Th1 and Th17 adaptive immune responses and the inhibition of the Th2 and Treg subpopulations seem to provide an ineffective defense against the development of a chronic *S. aureus* biofilm infection. Although somewhat limited, studies of CD4 T cell-mediated responses to *S. aureus* infections in humans have mirrored the studies presented herein. A recent study of patients with chronic rhinosinusitis, a biofilm infection of the sinus mucosa that is often due to *S. aureus*, demonstrated that there is a robust local Th1 response at the site of infection in these patients, as indicated by elevated levels of IFN-γ and MIP-1β (20). In addition, it has also been demonstrated that 34% of patients with atopic dermatitis have skin lesions containing alpha-toxin-producing strains of *S. aureus* and that sublytic levels of *S. aureus* alpha-toxin are capable of activating T cells and increasing IFN-γ production, leading to chronic disease (7). The protective role of the Treg lineage during *S. aureus* infection has also not been well elucidated, although there is evidence that children with immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, caused by mutations in the *FOXP3* gene and resulting in a lack of functional Tregs, are susceptible to *S. aureus* sepsis, due mainly to catheter-related infections (19). One reason may be that the tissue damage caused by proinflammatory cytokines may augment the ability of *S. aureus* to form biofilms on areas of devitalized tissue and vascular insufficiency. While this may point to a potential mechanism by which *S. aureus* successfully eludes clearance by the host immune system when progressing from an acute to chronic biofilm infection, more research is warranted. Lastly, although the results described above using the Th1-biased C57BL/6 mice show a strong correlation to chronic disease in patients, these data may not be replicated in other mouse strains such as Th2-biased BALB/c mice. Comparative studies using these two disparate mouse strains are presently ongoing in our laboratory.

Once an implant has become colonized with *S. aureus* and chronic infection develops, the only effective and curative treatment option available is removal of the infected implant (30, 31, 40). This procedure is both costly and traumatic to the patient. Better understanding of the host-adaptive immune response to *S. aureus* biofilm-mediated implant infection in the studies herein may lead to the development of more effective therapeutics and prophylaxes for these types of infections. In addition, these findings may lead to immune adjuvant therapy that will enable the manipulation of the host immune system, either alone or in combination with antimicrobial therapy, to promote the effective clearance of *S. aureus*.

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